# Avian Diseases

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# STUDIES OF NEWCASTLE DISEASE. II. EVALUATION OF TWO KILLED NEWCASTLE DISEASE VACCINES

By D. H. LEGENHAUSEN* and R. J. SINKIEWICZ**

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Received 12 May 1958

IN recent years the popularity of "live" virus Newcastle disease vaccines has steadily increased, while the percentage of birds immunized with "killed" vaccines has decreased. This trend is due, in part, to the ease with which live vaccines can be administered, as well as to the early reports regarding the low-grade immunity engendered by the use of inactivated vaccines.[1] Although the present widespread use of live-virus vaccines has served to minimize the losses resulting from Newcastle disease, the inherent nature of products of this type limits the possibility of eventual eradication of the disease. For this reason, investigations of inactivated Newcastle disease vaccines have been continued.

The authors have in the past completed two field studies dealing with the efficacy of inactivated Newcastle disease vaccines. Schoening *et al.*[7] found that although vaccination with a formalin-inactivated, commercially produced Newcastle disease vaccine did not entirely prevent the disease, it enabled the vaccinated birds to withstand a severe exposure, with relatively few losses as compared with unvaccinated control birds. Legenhausen *et al.*[6] have shown that an inactivated crystal violet vaccine was capable of stimulating a degree of immunity comparable to that resulting from the use of live virus vaccines under field conditions.

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* Now with the Animal Disease Eradication Division.
** Resigned.

The following experiments were designed to determine if the field observations could be confirmed under controlled laboratory conditions and to expand the presently available information relating to the duration of immunity following the use of inactivated Newcastle disease vaccines.

## MATERIALS AND METHODS

*Experimental birds.* The majority of chickens used in this study were standard bred New Hampshires obtained from the Animal Husbandry Research Division, Agricultural Research Service, U. S. Department of Agriculture. A small number of Rhode Island crosses from the same source were mixed with the New Hampshire chicks when they were placed under the electric brooder at 1 day of age. All chicks were from dams that had been vaccinated with live-virus, wing-web Newcastle disease vaccine at 14 to 16 weeks of age. Throughout the course of this study, the chickens were housed in concrete-floored, frame buildings, measuring 15 × 25 feet. A litter of wood chips or sawdust was maintained at all times.

*Strain of Newcastle disease virus.* The GB-Texas strain of Newcastle disease virus was employed in most phases of this work. Amnioallantoic fluid from the eighth embryo passage of this strain was used as a "seed" virus in the preparation of the experimental crystal violet vaccine, in the hemagglutination-inhibition (HI) and serum-neutralization (SN) tests, and in postvaccination challenge.

*Newcastle disease vaccines.* Two inactivated Newcastle disease vaccines were employed in this study. The first, an experimental vaccine, prepared according to the method of Doyle and Wright,[3] was made with the GB-Texas strain of Newcastle disease virus. This strain was found to be the most antigenic of three strains tested by Osteen.[7] The vaccine consisting of an 80 percent suspension of harvested embryos, membranes, and fluids in a 20 percent crystal violet solution (1 gram of crystal violet in 400 ml of ethylene glycol) was inactivated by 36 days' incubation at 37.5 C. The second vaccine, an oil emulsified, ultraviolet irradiated vaccine prepared with a strain of virus other than GB-Texas, was obtained from a commercial source.

*Isolation facilities.* The production of the experimental vaccine, as well as serological and challenge studies employed to evaluate it, were performed in the Newcastle disease building of the Animal Disease and Parasite Research Division. This building, a sealed unit, was designed for work with virus diseases of poultry and, as such, is constructed to prevent the spread of disease from room to room or from the building to the outside. The 50 percent bird-lethal-dose titrations ($BLD_{50}$), conducted in conjunction with all exposure studies, were performed in Horsfall-Bauer units in this building.

## EXPERIMENTAL PROCEDURES

When the chicks were 10 days old, they were divided into three main groups and placed in separate houses. Two groups of approximately 500 chicks each received the inactivated vaccines, while a third group of 600 birds was maintained as unvaccinated controls.

Two hundred and fifty chicks were given a single injection of 1.0 ml of the experimentally prepared crystal violet vaccine at 10 days of age. The remaining 250 chicks received two injections of vaccine—0.5 ml at 10 days of age and 1.0 ml 32 days later. All injections were made into the muscles of the leg. The birds receiving the single and double injections of vaccine were housed in one unit separated by a wire partition.

The commercially produced inactivated vaccine was handled in much the same manner as the crystal violet vaccine. The size of inoculation and route of administration were altered to conform to the manufacturer's recommendations. The birds receiving a single injection of vaccine were given 0.5 ml in the subcutaneous tissue of the neck. The birds receiving two injections were given 0.5 ml at 10 days of age and 1.0 ml 32 days later by the same route.

*Serological tests.* Prevaccination blood samples were obtained from randomly selected birds just prior to the administration of the inactivated vaccine at 10 days of age. The blood samples were pooled and allowed to clot. The serum was collected, filtered through a Boerner centrifugal filter, and

maintained at refrigerator temperature until tested. Post-vaccination blood samples, obtained at weekly intervals from five birds from each of the four groups of vaccinated birds and from the control birds, were handled in an identical manner. HI and SN tests were conducted on all samples.[8]

*Challenge exposure.* A lot of 30 birds from each of the four groups receiving either a single or double injection of the two inactivated vaccines, as well as from the control group, was brought to the Newcastle disease building at periodic intervals for challenging with the GB-Texas strain of Newcastle disease virus. Ten birds from each lot were placed in each of three separate rooms, where they were given an intramuscular injection of 0.1 ml of $10^{-3}$, $10^{-5}$, and $10^{-7}$ dilutions of the challenge virus, respectively.

In the initial challenge test when the birds were 6 weeks old (at the time when the second injection of vaccine was given to the birds receiving two injections), the vaccinated birds received 0.1 ml of a $10^{-3}$, $10^{-5}$, or $10^{-8}$ dilution of virus, whereas the controls received only the last dilution. Inasmuch as the $10^{8}$ dilution contained less than one $BLD_{50}$ of virus, it could not be considered a significant challenge. This perhaps was due to a faulty vial of virus. Four tightly sealed vials of virus were pooled, and dilutions of $10^{-3}$, $10^{-5}$, and $10^{-7}$ were employed in challenging all groups in subsequent exposures.

## EXPERIMENTAL RESULTS

SN and HI tests were conducted on serum samples obtained from vaccinated and control birds at weekly intervals. The results of these tests are presented in table 1. The blank spaces, indicating no test, are the results of either insufficient or contaminated serum.

The results of challenge exposure studies of the birds receiving single or double injections of either the crystal violet or ultraviolet irradiated vaccines are presented in table 2. The results of identical tests on the unvaccinated control birds are also incorporated in this table.

TABLE I

Serological test results

| Age in weeks | Crystal violet vaccine | | | | Commercial vaccine | | | | Controls | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Double vaccination | | Single vaccination | | Double vaccination | | Single vaccination | | | |
| | HI | SNI | HI | SNI | HI | SNI | HI | SNI | HI | SNI |
| 1 1/2 | 160 | — | 160 | — | 160 | — | 160 | — | 160 | — |
| 3 | 40 | 1.3 | 80 | 2.8 | 80 | 2.3 | 40 | 2.3 | 40 | 1.6 |
| 4 | 20 | — | 40 | — | 20 | — | 20 | — | 10 | — |
| 5 | 20 | — | 10 | 1.3 | 10 | 1.6 | 10 | 1.6 | 10 | 1.3 |
| 6 | 10 | — | 20 | 1.3 | 20 | 1.3 | 20 | 1.3 | 5 | 1.3 |
| 7 | 1280 | 5.3 | 10 | — | 1280 | 5.3 | 320 | 3.1 | 10 | — |
| 8 | 640 | 4.6 | 10 | 1.3 | 640 | 4.6 | 20 | 1.6 | 10 | 2.1 |
| 9 | 640 | 5.3 | 10 | 2.3 | 640 | 4.6 | 10 | — | 10 | 1.5 |
| 10 | 160 | 4.6 | 10 | 2.9 | 640 | 4.6 | 10 | — | 10 | 1.3 |
| 11 | 160 | 4.6 | 10 | — | 80 | — | 5 | — | 10 | 1.3 |
| 12 | 80 | — | 5 | 2.5 | 320 | — | 10 | 2.0 | 10 | — |
| 13 | 1280 | — | 10 | — | 1280 | — | 20 | — | 10 | 2.0 |
| 14 | 80 | — | 5 | 1.0 | 80 | — | 10 | 1.0 | 10 | 1.0 |
| 15 | 40 | 2.5 | 10 | 2.5 | 320 | — | 20 | 2.0 | 10 | 1.5 |
| 16 | 80 | — | 10 | 1.5 | 80 | 4.3 | 20 | — | 10 | 1.5 |
| 17 | 40 | 3.8 | 10 | 1.5 | 640 | 4.5 | 5 | 1.5 | 10 | 1.5 |
| 18 | 20 | 2.8 | 5 | 1.5 | 160 | 4.5 | 5 | 1.8 | 5 | 2.0 |
| 19 | — | — | — | — | — | — | — | — | — | — |
| 20 | 80 | 2.0 | 10 | 1.0 | 1280 | 4.5 | 160 | 3.3 | 0 | 1.0 |
| 21 | 40 | 3.3 | — | 1.0 | 640 | 4.5 | 20 | 2.5 | — | 1.0 |
| 22 | — | — | — | — | — | — | — | — | — | — |
| 23 | 20 | 1.5 | 10 | 1.0 | 160 | 3.3 | 40 | 3.5 | 10 | 1.0 |
| 24 | — | — | — | — | — | — | — | — | — | — |
| 25 | 10 | 3.5 | 10 | 1.0 | 640 | 4.5 | 0 | 1.0 | 5 | 1.0 |
| 26 | 10 | 1.7 | 10 | 1.0 | 320 | 4.0 | 10 | 1.0 | 5 | 1.0 |
| 36* | — | 2.0 | — | 3.9 | 320 | 3.5 | — | 4.0 | — | 1.9 |
| 56* | — | 2.0 | — | 3.9 | — | 3.9 | — | 4.0 | — | 2.4 |

HI = hemagglutination-inhibition titer of serum (BAI method[9]); SNI = serum-neutralization index.

* All singly vaccinated birds received a booster injection of vaccine when they were 34 weeks old.

TABLE II

Results of challenge exposure tests

| Age in weeks | $BLD_{50}$ | Crystal violet vaccine | | | | | | Commercial vaccine | | | | | | Controls | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Double vaccination | | | Single vaccination | | | Double vaccination | | | Single vaccination | | | | | |
| | | Log of reciprocal of virus dilutions employed in challenge exposure | | | | | | | | | | | | | | |
| | | 7 | 5 | 3 | 7 | 5 | 3 | 7 | 5 | 3 | 7 | 5 | 3 | 7 | 5 | 3 |
| 6* | | — | 10† | 40 | — | 70 | 40 | — | 60 | 60 | — | 60 | 60 | 80* | — | — |
| 10 | $10^{-7}$=14 | 100 | 100 | 100 | 50 | 20 | 70 | 100 | 90 | 100 | 40 | 40 | 30 | 10 | 0 | 0 |
| 14 | $10^{-7}$=17 | 100 | 90 | 70 | 40 | 60 | 0 | 100 | 100 | 100 | 60 | 10 | 80 | 10 | 20 | 0 |
| 18 | $10^{-7}$=10 | 100 | 100 | 90 | 40 | 80 | 40 | 100 | 70 | 80 | 50 | 50 | 60 | 40 | 10 | 30 |
| 26 | $10^{-7}$=1 | 100 | 100 | 80 | 60 | 60 | 60 | 90 | 90 | 90 | 70 | 80 | 70 | 20 | 0 | 40 |
| 36‡ | $10^{-5}$=17 | | 100 | — | — | 80 | — | — | 90 | — | — | 100 | — | — | 50 | — |
| 56‡ | $10^{-5}$=14 | — | — | 90 | — | — | 100 | — | — | 80 | — | — | 80 | — | — | 30 |

* Virus dilutions of $10^{-8}$, $10^{-5}$, $10^{-3}$ were used in the 6-week test.
† Percentage of protection.
‡ All singly vaccinated birds received a booster injection of vaccine when they were 34 weeks old.

## DISCUSSION

The experiments described in this paper demonstrate the degree and duration of immunity following the use of a single or divided dose of two inactivated Newcastle disease vaccines. None of the birds receiving a single injection of either the experimentally prepared crystal violet vaccine or the commercially produced ultraviolet irradiated vaccine acquired a satisfactory immunity, as indicated by challenge-exposure studies at 6, 10, 14, 18, 26, 36, and 56 weeks of age. These results were partially substantiated by serological tests. In general, birds receiving only a single injection of vaccine or maintained as unvaccinated controls failed to exhibit a significant SN or HI titer beyond the third week of age. Two exceptions to the foregoing generality were found when the sera obtained at 7 and 20 weeks of age from chicks receiving a single injection of the commercially produced vaccine were tested. These results emphasized the danger of basing final interpretations entirely upon a single serological examination.

Birds that were given a second, or booster, injection of vaccine exhibited an increased resistance to intramuscular challenge when exposed to the GB-Texas strain of Newcastle disease virus throughout the course of this 13 month experiment. When the birds were challenged at 10 and 14 weeks of age (4 and 8 weeks after the second injection of vaccine), a decided difference in the percentage of protection was noted. The birds receiving two injections of vaccine showed 70 to 100 percent protection, whereas the controls showed a variable degree of protection ranging from 0 to 20 percent. An immunity of this duration would provide adequate protection throughout the life span of most broilers. Although challenge exposures when the birds were 18, 26, 36, and 56 weeks of age serve mainly to demonstrate the duration of immunity, it should be noted that a booster injection of vaccine when the birds receiving single injections were 34 weeks old resulted in an increased resistance to challenge. In doubly vaccinated birds an increase in SN and HI titers was also observed. Birds receiving two injections of commercially produced, ultraviolet irradiated vaccine retained significant HI and SN titers much

longer than did those receiving two doses of crystal violet vaccine. On the other hand, the birds that were given two injections of the crystal violet vaccine exhibited a greater degree of resistance when challenged. The increased resistance to the challenge exposures employed in birds vaccinated with crystal violet vaccine may reflect the use of a homologous strain of Newcastle disease virus in both the production of the crystal violet vaccine and the challenge exposures employed to evaluate it.

The variation of the percentage of protection in the control birds in these tests cannot be adequately explained. Biological error, as well as age resistance, may account for the noted deviation.

In examining the results of the challenge-exposure studies, it will be noted that the percentage of protection of the vaccinated and control birds is not dependent upon the dilution of virus employed. It appears to make little difference whether a challenged bird received 100 or 100,000 $BLD_{50}$ of Newcastle disease virus.

The increased resistance to challenge following the use of a second, or booster, injection of inactivated Newcastle disease vaccine has previously been reported by a number of investigators. In 1946, Brandly *et al.*,[2] after studies of a formalinized vaccine, reported that divided doses of vaccine when administered at intervals in excess of 1 week proved more effective than a single dose of the same total quantity. In more recent work these findings have been confirmed by Waller and Gardiner,[9] and extended by Hofstad,[4,5] who has emphasized the importance of the time interval between doses in establishing the maximum immune response.

## SUMMARY

The results of studies designed to evaluate an experimentally prepared crystal violet Newcastle disease vaccine and a commercially produced, ultraviolet-irradiated Newcastle disease vaccine are presented. Single and double vaccinations were performed with each product. A single injection of either vaccine at 10 days of age failed to produce an effective im-

munity against Newcastle disease, as measured by serological and challenge-exposure studies. A booster injection of vaccine 32 or 228 days after the initial vaccination produced a decided increase in resistance to intramuscular challenge exposures to the GB-Texas strain of Newcastle disease virus. In general, these findings were substantiated by the serological tests.

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# STUDIES OF NEWCASTLE DISEASE. III. FURTHER STUDIES OF A KILLED NEWCASTLE DISEASE VACCINE

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IN the previous paper[5], it was shown that satisfactory immunity throughout a broiler-growing period could be obtained by the use of two injections of a killed Newcastle disease vaccine. It is the purpose of this paper to report on further explorations of the protection afforded by a vaccination program of this type, with special emphasis on the effect of variations in dosage and time intervals between injections. Additional information was also sought on the degree of immunity that exists between the time of the primary and secondary injections of vaccine.

## MATERIALS AND METHODS

*Chickens.* The chicks used in this study were standard-bred Rhode Island Reds obtained from the Animal Husbandry Research Division of the U. S. Department of Agriculture. The chicks were from five weekly hatches of the same flock and were from dams that had been vaccinated with a live-virus, wing-web Newcastle disease vaccine at about 3 months of age. Throughout the course of this study, the chicks were housed in concrete-floored, frame buildings, measuring 15 $\times$ 25 feet. A sawdust litter was maintained at all times.

* Now with the Animal Disease Eradication Division.
** Resigned.

*Isolation facilities.* The experimental vaccine was produced and evaluated in the Newcastle disease building of the Animal Disease and Parasite Research Division. The building was designed for work with virus diseases of poultry and, as such, is constructed to prevent the spread of disease from room to room or from the building to the outside.

*Virus.* Amnioallantoic fluids from the eighth embryo passage of the GB-Texas strain of Newcastle disease virus were used exclusively in this work.

*Vaccine.* The experimentally prepared, inactivated crystal violet vaccine described in part II of this study was used.

## EXPERIMENTAL PROCEDURES

Parentally immune chicks, which were received from the Animal Husbandry Research Division, Beltsville, Md., at a given time, served as the vaccinated principals and untreated controls in one of the various vaccination programs conducted during the course of these trials. The first hatch was used to determine the immunizing effect produced by varying the size of a single injection of vaccine, whereas the four other hatches were subdivided to determine the effect of variations in both the total quantity of vaccine and the time interval employed between injections in a two-dose vaccination program. The chicks were placed in 10 major groups and were housed in similar quarters. Nine of these groups, serving as principals, were given either a single or double injection of vaccine, while birds in the tenth group were maintained as unvaccinated controls.

*Group A, singly vaccinated principals.* One hundred chicks from the first hatch received only single injections of varying amounts of vaccine when they were 10 days of age. Five lots of 20 birds each were given intramuscular injections of 0.1, 0.25, 0.5, 1.0, and 2.0 ml doses, respectively (see table 1).

*Groups B, C, D, E, doubly vaccinated principals (small primary dose).* Three hundred chicks from the four other hatches were divided into four groups (B, C, D, and E) of five lots each, with 15 birds to each lot. The chicks were each

given a small (0.1 ml) intramuscular injection of vaccine at 10 days of age, and at one of four weekly intervals, the five lots in a single group were given second injections of vaccine in doses of 0.1, 0.25, 0.5, 1.0, and 2.0 ml, respectively (table 2).

*Groups F, G, H, I, doubly vaccinated principals (large primary dose).* Another 300 chicks from the four hatches were likewise divided into four groups (F, G, H, and I) of five lots each, with 15 birds to each lot. These chicks were each given a large (2.0 ml) intramuscular injection of vaccine at 10 days of age. Each group was then revaccinated in an identical manner to the birds in groups B, C, D, and E (table 3).

*Unvaccinated controls.* A portion of each of the five hatches was maintained as unvaccinated controls. Birds from each hatch were subjected to the same challenge exposure that their singly and doubly vaccinated hatch mates received throughout the course of these trials.

*Serological tests.* Blood samples were obtained from 10 to 15 randomly selected birds in each group just prior to the administration of the first and second injections of vaccine and from 5 birds in each lot just before the birds were challenged. The blood samples from each group or lot were pooled and allowed to clot. The sera were collected, filtered through Boerner centrifugal filters, and held at refrigerator temperature until tested. All samples were subjected to both the hemagglutination-inhibition (HI) and serum-neutralization (SN) tests.

*Challenge exposure.* Each lot of birds that received either a single or double injection of vaccine, along with a like number of unvaccinated controls, was brought to the Newcastle disease building 3 weeks after the final injection of vaccine for challenge-exposure studies.

Ten birds from each lot that received a booster injection of varying amounts of vaccine were placed in separate cages where they were challenged by means of an intramuscular injection of 0.1 ml of a $10^{-6}$ dilution of amnioallantoic fluid containing the GB-Texas strain of the Newcastle disease virus.

TABLE I

Results of serological and challenge-exposure studies:
Singly vaccinated principals

| Group | Lot No. | Vaccination schedule | | | Serum titers | | | | Challenge exposure | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Prevaccination | | Prechallenge | | | Percentage of protection | |
| | | Birds per lot | Age in days | Dose (ml) | SNI | HI | SNI | HI | Chall. dose* ($BLD_{50}$) | Vacc. | Controls |
| A | 1 | 20 | 10 | 0.1 | 4.0 | 2560 | 0 | 10 | 1.7 | 60 | 20 |
| | 2 | 20 | 10 | 0.25 | 4.0 | 2560 | 0 | 10 | 1.7 | 50 | 20 |
| | 3 | 20 | 10 | 0.5 | 4.0 | 2560 | 0.7 | 10 | 1.7 | 80 | 20 |
| | 4 | 20 | 10 | 1.0 | 4.0 | 2560 | 0.7 | 10 | 1.7 | 100 | 20 |
| | 5 | 20 | 10 | 2.0 | 4.0 | 2560 | 1.7 | 5 | 1.7 | 100 | 20 |

SNI = serum-neutralization index; HI = hemagglutination-inhibition titer of serum (BAI method).

* Number of $BLD_{50}$ contained in challenge dose. $BLD_{50}$ of challenge strain determined in nonimmune hatch mates.

Birds receiving only a single injection of varying amounts of vaccine were challenged in a similar manner with a $10^{-7}$ dilution of this virus suspension. In all cases the challenge strain of virus was titered in untreated control birds of the same age.

## EXPERIMENTAL RESULTS

The results of serological and challenge-exposure studies on the five lots of birds receiving only a single injection of varying amounts of vaccine at 10 days of age are presented in table 1.

The results of serological and challenge-exposure studies on the 20 lots of birds that received a small initial injection of vaccine at 10 days of age and booster injections of varying amounts at intervals of 1, 2, 3, or 4 weeks are presented in table 2.

Results of serological and challenge-exposure studies on the 20 lots of birds that received a large initial injection of vaccine at 10 days of age and booster injections of varying amounts at intervals of 1, 2, 3, or 4 weeks are presented in table 3.

## DISCUSSION

Although comparatively large doses (2.0 ml) of inactivated Newcastle disease vaccine were administered to a high percentage of the vaccinated principals employed in these trials, postvaccination reactions were never observed during the intervals between the time of vaccination and challenge. When the singly vaccinated birds in group A were challenged at 31 days of age (3 weeks postvaccination), a definite correlation between the size of the dose and the degree of resistance to challenge was noted. The two lots (1 and 2) that received single injections of 0.1 and 0.25 ml of vaccine at 10 days of age exhibited 60 and 50 percent protection, respectively, against a challenge-exposure dose that killed 80 percent of the unvaccinated controls. The three lots (3, 4, and 5) that were given larger single injections of 0.5, 1.0, and 2.0 ml of vaccine at 10 days of age exhibited 80, 100, and 100 percent protection, respectively, when exposed to the identical challenge.

## TABLE II

Results of serological and challenge-exposure studies:
Doubly vaccinated principals
(small primary dose)

| Group | Lot No.* | Vaccination schedule: Age in days: 10 — 1st vacc. | 17 — 2nd vacc | 24 — 2nd vacc. | 31 — 2nd vacc. | 38 — 2nd vacc. | Serum titers: Before— 1st vacc. SNI | 1st vacc. HI | 2nd vacc. SNI | 2nd vacc. HI | Chall. SNI | Chall. HI | Challenge exposure: Chall. dose† ($BLD_{50}$) | Percentage of protection: Vacc. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B | 6 | 0.1 | 0.1 | | | | 3.7 | 2560 | 3.2 | 40 | 1.2 | 5 | 150 | 30 | 0 |
| | 7 | 0.1 | 0.25 | | | | 3.7 | 2560 | 3.2 | 40 | 1.0 | 5 | 150 | 80 | 0 |
| | 8 | 0.1 | 0.5 | | | | 3.7 | 2560 | 3.2 | 40 | 1.0 | 20 | 150 | 90 | 0 |
| | 9 | 0.1 | 1.0 | | | | 3.7 | 2560 | 3.2 | 40 | 2.7 | 320 | 150 | 90 | 0 |
| | 10 | 0.1 | 2.0 | | | | 3.7 | 2560 | 3.2 | 40 | 3.0 | 320 | 150 | 100 | 0 |
| C | 11 | 0.1 | | 0.1 | | | 3 7 | 2560 | 1.0 | 5 | 1.5 | 20 | 120 | 90 | 0 |
| | 12 | 0.1 | | 0.25 | | | 3.7 | 2560 | 1.0 | 5 | 1.5 | 20 | 120 | 90 | 0 |
| | 13 | 0.1 | | 0.5 | | | 3.7 | 2560 | 1.0 | 5 | 3.0 | 160 | 120 | 90 | 0 |
| | 14 | 0.1 | | 1.0 | | | 3.7 | 2560 | 1.0 | 5 | 3.0 | 160 | 120 | 100 | 0 |
| | 15 | 0.1 | | 2.0 | | | 3.7 | 2560 | 1.0 | 5 | 4.0 | 1280 | 120 | 100 | 0 |
| D | 16 | 0.1 | | | 0.1 | | 3.7 | 320 | 1.5 | 10 | 1.0 | 10 | 180 | 80 | 10 |
| | 17 | 0.1 | | | 0.25 | | 3.7 | 320 | 1.5 | 10 | 1.0 | 10 | 180 | 90 | 10 |
| | 18 | 0.1 | | | 0.5 | | 3.7 | 320 | 1.5 | 10 | 4.0 | 1280 | 180 | 90 | 10 |
| | 19 | 0.1 | | | 1.0 | | 3.7 | 320 | 1.5 | 10 | 4.5 | 1280 | 180 | 100 | 10 |
| | 20 | 0.1 | | | 2.0 | | 3.7 | 320 | 1.5 | 10 | 4.5 | 1280 | 180 | 100 | 10 |
| E | 21 | 0.1 | | | | 0.1 | 4.2 | 160 | 1.0 | 10 | 1.2 | 0 | 160 | 100 | 0 |
| | 22 | 0.1 | | | | 0.25 | 4.2 | 160 | 1.0 | 10 | 2.3 | 5 | 160 | 100 | 0 |
| | 23 | 0.1 | | | | 0.5 | 4.2 | 160 | 1.0 | 10 | 4.5 | 320 | 160 | 100 | 0 |
| | 24 | 0 1 | | | | 1.0 | 4.2 | 160 | 1.0 | 10 | 4.5 | 640 | 160 | 100 | 0 |
| | 25 | 0.1 | | | | 2.0 | 4.2 | 160 | 1.0 | 10 | 4 5 | 1280 | 160 | 100 | 0 |

SNI = serum-neutralization index; HI = hemagglutination-inhibition titer of serum (BAI method).

* Ten of the 15 birds in each of these lots were challenged.

† Number of $BLD_{50}$ contained in challenge dose. $BLD_{50}$ of challenge strain determined in nonimmune hatch mates.

## TABLE III

Results of serological and challenge-exposure studies:
Doubly vaccinated principals
(large primary dose)

| Group | Lot No.* | Vaccination schedule: Age in days 10 — 1st vacc | 17 — 2nd vacc. | 24 — 2nd vacc. | 31 — 2nd vacc. | 38 — 2nd vacc. | Serum titers: Before 1st vacc. SNI | 1st vacc. HI | Before 2nd vacc. SNI | 2nd vacc. HI | Before Chall. SNI | Chall. HI | Challenge exposure: Chall. dose† ($BLD_{50}$) | Percentage of protection: Vacc. | Controls |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F | 26 | 2.0 | 0.1 | | | | 3.7 | 2560 | 1.7 | 20 | 1.0 | 20 | 150 | 90 | 0 |
| | 27 | 2.0 | 0.25 | | | | 3.7 | 2560 | 1.7 | 20 | 1.5 | 40 | 150 | 80 | 0 |
| | 28 | 2.0 | 0.5 | | | | 3.7 | 2560 | 1.7 | 20 | 2.5 | 40 | 150 | 100 | 0 |
| | 29 | 2.0 | 1.0 | | | | 3.7 | 2560 | 1.7 | 20 | 2.5 | 320 | 150 | 100 | 0 |
| | 30 | 2.0 | 2.0 | | | | 3.7 | 2560 | 1.7 | 20 | 5.5 | 1280 | 150 | 100 | 0 |
| G | 31 | 2.0 | | 0.1 | | | 3.7 | 2560 | 1.0 | 5 | 3.0 | 80 | 120 | 90 | 0 |
| | 32 | 2.0 | | 0.25 | | | 3.7 | 2560 | 1.0 | 5 | 3.7 | 80 | 120 | 100 | 0 |
| | 33 | 2.0 | | 0.5 | | | 3.7 | 2560 | 1.0 | 5 | 2.5 | 80 | 120 | 80 | 0 |
| | 34 | 2.0 | | 1.0 | | | 3.7 | 2560 | 1.0 | 5 | 3.7 | 640 | 120 | 100 | 0 |
| | 35 | 2.0 | | 2.0 | | | 3.7 | 2560 | 1.0 | 5 | 5.4 | 1280 | 120 | 90 | 0 |
| H | 36 | 2.0 | | | 0.1 | | 3.7 | 320 | 1.5 | 40 | 1.5 | 5 | 180 | 100 | 10 |
| | 37 | 2.0 | | | 0.25 | | 3.7 | 320 | 1.5 | 40 | 2.3 | 10 | 180 | 90 | 0 |
| | 38 | 2.0 | | | 0.5 | | 3.7 | 320 | 1.5 | 40 | 4.0 | 320 | 180 | 100 | 10 |
| | 39 | 2.0 | | | 1.0 | | 3.7 | 320 | 1.5 | 40 | 3.5 | 640 | 180 | 100 | 10 |
| | 40 | 2.0 | | | 2.0 | | 3.7 | 320 | 1.5 | 40 | 4.5 | 1280 | 180 | 100 | 10 |
| I | 41 | 2.0 | | | | 0.1 | 4.2 | 160 | 2.8 | 10 | 4.0 | 320 | 160 | 100 | 0 |
| | 42 | 2.0 | | | | 0.25 | 4.2 | 160 | 2.8 | 10 | 3.8 | 640 | 160 | 100 | 0 |
| | 43 | 2.0 | | | | 0.5 | 4.2 | 160 | 2.8 | 10 | 3.5 | 640 | 160 | 100 | 0 |
| | 44 | 2.0 | | | | 1.0 | 4.2 | 160 | 2.8 | 10 | 3.8 | 1280 | 160 | 100 | 0 |
| | 45 | 2.0 | | | | 2.0 | 4.2 | 160 | 2.8 | 10 | 4.5 | 640 | 160 | 100 | 0 |

SNI = serum-neutralization index; HI = hemagglutination-inhibition titer of serum (BAI method).

* Ten of the 15 birds in each of these lots were challenged.

† Number of $BLD_{50}$ contained in challenge dose. $BLD_{50}$ of challenge strain determined in nonimmune hatch mates.

Although a small amount of vaccine did not provide adequate protection when the birds were challenged 3 weeks after vaccination, it served as an effective immunogenic stimulus when combined with larger booster injections of vaccine at optimum time intervals in a two-dose vaccination program (table 2).

In the four groups of birds (B, C, D, and E) that were given a small initial dose of vaccine at 10 days of age and a booster injection of varying amounts of vaccine at intervals of 1, 2, 3, or 4 weeks, the resulting immunity tended to be proportional to both the total quantity of vaccine administered in two doses and to the time interval employed between the primary and secondary injections of vaccine. Only 1 of the 20 lots of birds that received a small primary injection of vaccine failed to show a satisfactory degree of resistance when challenged. This lot (no. 6) received a second 0.1 ml booster injection of vaccine 1 week after it had been given an identical primary inoculation. This illustrates the inability of small amounts of vaccine, when administered in a single injection or subdivided and administered in dual injections at short intervals, to stimulate a satisfactory state of immunity. The complete protection should be noted of the four lots of birds (nos. 10, 15, 20, and 25) receiving the largest total quantity of vaccine and of the five lots of birds (nos. 21-25) in group E where 4 weeks were allowed to elapse between the first and second injections of vaccine.

All groups of birds (F, G, H, and I) that were given a large initial dose of vaccine at 10 days of age and a booster injection of varying amounts of vaccine at intervals of 1 to 4 weeks exhibited a significant degree of resistance when challenged. The maximum immune response again appeared to be related to both the total quantity of vaccine used and the time interval employed. The protection afforded by the largest total quantity of vaccine (lots 30, 35, 40, and 45) and the greatest time interval until revaccination (lots 41-45) should be noted.

The increased protection afforded doubly vaccinated birds when a large primary injection of vaccine was employed,

as well as the total protection of all birds that were given the larger single injections of vaccines, illustrates the importance of the size of the initial dose of vaccine in a two-dose vaccination program. Although doubly vaccinated principals received an initial injection of either 0.1 or 2.0 ml of vaccine in this experiment, previous experience would favor an initial injection of either 0.5 or 1.0 ml.

The prevaccination serological titers obtained in all groups demonstrate the presence of a significant degree of parentally conferred immunity, the progressive decrease in HI titers in groups D, E, H, and I may indicate the depletion of this antibody in the dams or reflect the disadvantages of employing pooled sera samples in serological work. When examining prechallenge titers it should be noted that in no instance did a single injection of vaccine reverse the decline in parental immunity, as measured by SN and HI tests. It should also be noted that while three of the five lots of birds that received but a single injection of vaccine proved to be quite resistant to the challenge exposure employed, serological evidence of this immune state could not be demonstrated. A parallel situation exists in the case of the doubly vaccinated birds receiving small primary or secondary injections of vaccine. Although seven of the eight lots of birds that received a primary injection of 0.1 ml of vaccine and a booster injection of either 0.1 or 0.25 ml of vaccine were resistant to challenge, significant titers to both the SN and HI tests were lacking.

With a single exception, significant serum titers followed the use of the larger (0.5, 1.0, and 2.0 ml) booster injections of vaccines regardless of the size of the primary dose, when the two injections were given at intervals of 2 weeks or more. When booster injections of this size were given a week after the initial dose of vaccine was administered, the resulting serum titers were extremely variable, being generally proportional to the size of the booster injection of vaccine.

The resistance to challenge of serologically negative birds as noted in this experiment has previously been reported by a number of investigators.[1,2,6,7] We have observed

that the duration of immunity in serologically negative, singly vaccinated birds does not hold up without proper revaccination. Following such revaccination, the SN and HI titers increase. Although this would suggest that the postvaccination serological response may be used as a guide in anticipating the comparative duration of immunity in serologically negative and positive groups of birds, this presumption has yet to be confirmed or disproved through controlled laboratory studies.

## SUMMARY

The results of studies designed to determine the immune state following a single injection of varying amounts of a crystal violet Newcastle disease vaccine, as well as the immunizing effect resulting from variations in the total quantity of vaccine and time interval employed in a two-dose vaccination program, are presented.

The data obtained in these trials indicate that the quantity of vaccine administered in a single injection may exert a decided influence on the resulting state of immunity. A small, single injection of vaccine (0.1 or 0.25 ml) failed to provide adequate protection against the challenge exposure employed. Somewhat larger single doses of vaccine (0.5, 1.0, or 2.0 ml) produced increased refractivity to the challenge exposure employed 3 weeks after vaccination. Serological evidence of immunity was lacking regardless of the amount of vaccine administered in a single injection.

The results obtained by varying the time interval and total quantity of vaccine employed in a two-dose vaccination program indicate that both of these factors may materially influence the resulting state of immunity. Small amounts of vaccine administered in divided doses at short intervals failed to offer any advantages over a single injection of the same quantity. Larger amounts of vaccine administered at identical intervals afforded a significant increase in resistance to challenge that could be correlated serologically. The variable resistance to challenge observed in the groups of birds receiving like amounts of vaccine at different time intervals in a two-dose vaccination program demonstrates the im-

portance of the time factor in producing the maximum immune response. Although the response was not always proportional to the time interval employed, the occurrence of complete protection in all groups receiving a booster injection of vaccine 4 weeks after the primary inoculation is noteworthy. Four weeks was the longest interval employed in this study, and the optimum interval between the time of the first and second injections of vaccine may be well beyond this point as indicated by Hofstad.[3,4]

## CONCLUSIONS

Small quantities of inactivated Newcastle disease vaccine administered in a single dose or subdivided and administered in dual injections at short intervals failed to provide adequate immunity as gauged by serological and challenge-exposure studies. Although larger single injections of vaccine were capable of affording at least partial protection against challenge exposure, this immune state could not be demonstrated by serological procedures. Booster injections of vaccine, 4 weeks after the primary injection of vaccine (groups E and I), afforded the best protection against a tenfold increase in the challenge-exposure dose. Serological evidence of the heightened state of immunity following a booster injection of vaccine was demonstrated.

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# MEASUREMENT OF PATHOGENICITY OF TURKEY ORNITHOSIS AGENTS FOR MICE *

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CHARACTERIZATION of ornithosis and psittacosis isolates customarily are made by describing their morphology, their antigenic relationship to known psittacosis strains, their pathogenicity for guinea pigs, pigeons and rice birds, and their relative toxicity for mice.[2] The toxin titration in mice consists of injecting serial dilutions of a heavily infected yolk sac suspension of the isolate intravenously into mice and observing the effects produced in this host. Mice dying within 48 hours are considered to have succumbed to the effects of a toxin produced by the agent. Isolates of the pneumonitis-psittacosis lymphogranuloma venereum group from different species have often had surprisingly different toxin titers indicating that the amount of toxin produced by members of this group varies greatly. Toxins produced by different members of the group have been shown to be antigenically specific enabling classification of new isolates.[1]

In the course of isolation of ornithosis agents from California turkeys and New Jersey turkeys, it was noted that the isolates from the two regions differed in their pathogenicity for mice. Titrations of the yolk sac propagated ornithosis agents in mice, by the intravenous route, had shown that suspensions of the New Jersey isolate were highly toxic for

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this animal, while suspensions of the California isolate were of relatively low toxicity.[3] Titrations of these isolates in mice by the intraperitoneal route, however, indicated that the numerical difference between infectivity and lethality titers might be a useful reproducible index of pathogenicity of ornithosis and psittacosis agents for mice. The purpose of this report is to describe pathogenicity index determinations on three turkey ornithosis isolates, two psittacosis isolates and one pigeon ornithosis isolate.

## MATERIALS AND METHODS

The turkey ornithosis viruses were obtained from ornithosis outbreaks in New Jersey and California in 1954 and again in California in 1956. These viruses were labeled the NJ1, C1 and C2 ornithosis isolates.[4,5] The Mexican parrot isolation was made at the George Williams Hooper Foundation, San Francisco, by Dr. B. Eddie in 1955. All other isolations were made by the author at the George Williams Hooper Foundation and were performed as follows: several grams of suspect tissue were ground in a mortar with sand and diluted to a 20 percent suspension in antibiotic broth*. The suspension was allowed to stand overnight at 5 to 8C, and for primary isolation the supernatant was then injected in half milliliter amounts intraperitoneally into each of four white, 18 to 20 gram male Princeton mice. Mice dying or appearing sick within 15 days were examined for the presence of gross lesions of ornithosis and any suspicious exudate was stained by Macchiavello's method and examined microscopically for the presence of intracellular elementary bodies. At least three blind passages in mice were performed before a tissue was considered negative for the presence of ornithosis agents. The starting materials for the mouse pathogenicity titrations of the various isolates were spleens and livers of mice representing at least the second passage of original material. The mouse spleens and livers were ground as described above and the overnight supernatants were diluted decimally. Half milli-

* Consisting of tyrothricin, 20 micrograms/ml; streptomycin-HCl, 250 micrograms/ml. and sodium sulfadiazine, 500 micrograms/ml. in beef heart broth.

liter aliquots of each dilution were injected intraperitoneally into each of 10 mice. Animals that survived 21 days following injection were killed, examined for lesions of ornithosis, and spleens and livers from each animal were collected and passed individually in two mice to determine if any infective particles remained. Fifty percent endpoint determinations ($LD_{50}$ and $ID_{50}$) were calculated by the method of Reed and Muench.[6]

## RESULTS

The results of intraperitoneal titrations in mice of turkey ornithosis agents from three sources are presented in table 1. Determination of lethal and infectivity 50 percent endpoints indicated that each isolate had a different degree of pathogenicity for mice. Calculation of the log $ID_{50}$-$LD_{50}$ difference for each isolate resulted in a number, referred to by us as a pathogenicity index, which reflected the ability of the orni-

TABLE I

Mouse pathogenicity tests on ornithosis isolates of varying toxicity

| | Turkey Isolates | | | | | |
|---|---|---|---|---|---|---|
| | New Jersey isolate | | Selma, Calif. C-1 isolate | | Sheridan, Calif. C-2 isolate | |
| | Dead | Lesions | Dead | Lesions | Dead | Lesions |
| Log dilution | Inoc. | Inoc. | Inoc. | Inoc. | Inoc. | Inoc. |
| 0 | 10/10 | 10/10 | 10/10 | 10/10 | 0/10 | 10/10 |
| -1 | 10/10 | 10/10 | 4/10 | 10/10 | 0/10 | 8/10 |
| -2 | 10/10 | 10/10 | 5/10 | 10/10 | 0/10 | 5/10 |
| -3 | 10/10 | 10/10 | 5/10 | 10/10 | 0/10 | 10/10 |
| -4 | 10/10 | 10/10 | 10/10 | 10/10 | 0/10 | 10/10 |
| -5 | 10/10 | 10/10 | 0/10 | 4/10 | 0/10 | 4/10 |
| -6 | 4/10 | 6/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| -7 | 0/10 | 0/10 | 1/10 | 1/10 | 0/10 | 0/10 |
| -8 | 0/10 | 0/10 | 0/10 | 0/10 | | |
| -9 | 0/10 | 0/10 | 0/10 | 0/10 | | |
| -10 | 0/10 | 0/10 | | | | |
| Log $LD_{50}$ | 5.83 | | 3.00 | | 0.00 | |
| Log $ID_{50}$ | | 6.17 | | 4.92 | | 4.45 |
| Difference | | 0.34 | | 1.92 | | 4.45 |

Note: All surviving mice were killed and examined on the 21st day after infection.

thosis organism to grow, multiply, and kill mice. Titration of several infected tissues containing different quantities of the same agent indicated that the index remained constant for each isolate.

A low index number meant high mouse pathogenicity, for, as the lethality titer approached equivalence with the infectivity titer, the index approached zero. Conversely, a high index number, e.g. 4.00, indicated an infectivity 10,000 times greater than the lethality.

Indices of turkey isolates ranged from high to low. The psittacine and pigeon isolates (table 2) all appeared to be of relatively low pathogenicity.

The California isolates (when injected intraperitoneally) failed to kill mice regularly and often produced a lingering disease characterized by enlargement of the spleen and liver, accumulation of serofibrinous fluid in the peritoneal cavity

TABLE II

Mouse pathogenicity tests on psittacosis and ornithosis isolates of varying toxicity

| | Psittacine isolates | | | | Pigeon isolate | |
|---|---|---|---|---|---|---|
| | Mexican parrot isolate | | Pensacola, Fla. parakeet isolate | | Sheridan, Calif. pigeon isolate | |
| | Dead | Lesions | Dead | Lesions | Dead | Lesions |
| Log dilution | Inoc. | Inoc. | Inoc. | Inoc. | Inoc. | Inoc. |
| 0 | 0/10 | 10/10 | 10/10 | 10/10 | 0/8** | 8/8 |
| −1 | 0/8* | 6/8 | 6/10 | 10/10 | 0/10 | 8/10 |
| −2 | 0/10 | 9/10 | 2/10 | 10/10 | 0/10 | 9/10 |
| −3 | 0/9* | 8/9 | 5/10 | 10/10 | 0/10 | 10/10 |
| −4 | 0/10 | 10/10 | 1/10 | 10/10 | 0/10 | 10/10 |
| −5 | 0/10 | 4/10 | 1/10 | 10/10 | 0/10 | 4/10 |
| −6 | 0/10 | 2/10 | 1/10 | 6/10 | 0/10 | 0/10 |
| −7 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| −8 | 0/10 | 0/10 | 0/10 | 0/10 | | |
| −9 | 0/10 | 0/10 | 0/10 | 0/10 | | |
| Log $LD_{50}$ | 0.00 | | 1.86 | | 0.00 | |
| Log $ID_{50}$ | | 4.71 | | 6.17 | | 4.61 |
| Difference | 4.71 | | 4.31 | | 4.61 | |

* Two mice died with concurrent *B. piliformis* infection and were excluded from tabulation.

** Two mice died with concurrent enteritis and were excluded from tabulation.

in sufficient volume to give the animal a bloated appearance. Smears of this fluid generally showed few intracellular elementary bodies. In contrast, the New Jersey isolate invariably caused death within 5 to 10 days with little or no splenic or hepatic enlargement. A fibrinous peritonitis and pericarditis was generally present with severely hemorrhagic lungs. Smears of the peritoneal exudate usually showed numerous intracellular elementary bodies.

A pathogenicity index can be useful in characterizing new virus isolates, but it may find greater use in detecting changes in pathogenicity of these viruses. In the passage of disease agents of low pathogenicity through susceptible animals or birds, certain hosts may serve as selective media for mutant viruses of high pathogenicity or toxicity. Determination of a decrease in the log $ID_{50}$-$LD_{50}$ difference by titration of virus suspensions isolated from each successive animal passage would reveal the presence of a new population of pathogenic mutants.

## SUMMARY

Six ornithosis and psittacosis viruses were characterized in terms of mouse pathogenicity by the intraperitoneal route of inoculation. A pathogenicity index based on the log $ID_{50}$-log $LD_{50}$ difference was calculated for each isolate. This method, when applied with sufficient numbers of organisms, provided a means for the measurement of pathogenicity independent of the concentration of the infecting agent. Use of this test for detecting pathogenicity changes in viral populations was suggested.

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# EGG TRANSMISSION OF INFECTIOUS SINUSITIS IN NATURALLY INFECTED TURKEYS*†

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SEVERAL investigators have demonstrated that infectious sinusitis can be egg transmitted.[5,6,7,8,10] Grumbles and Boney[3] encountered field evidence that suggested egg transmission of infectious sinusitis, but they were unable to demonstrate this mode of transmission experimentally. A report of egg transmission of a respiratory disease of undetermined etiology by Harr[4] shows some resemblance to infectious sinusitis. A field report in the lay press by Blom[2] on egg transmission of "air sac infection" may also refer to infectious sinusitis.

This paper reports, 1) observations on egg transmission of infectious sinusitis to poults from infected breeders of a commercial hatchery, and 2) results of an attempt to maintain naturally infected and noninfected stock from year to year.

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## MATERIALS AND METHODS

*Experimental turkeys.* Nine commercial supply flocks of broad breasted bronze turkeys were selected to produce ten experimental lots of poults for brooding in 1954. The commercial supply flocks had been visited in April, 1954, and classified as infected or noninfected with infectious sinusitis. The basis for classification was the presence or absence of symptoms at the time of the visit and the history of the flock as determined from the owner and hatchery representative or hatchery representative alone. Table 1 summarizes information on the hatchery supply flocks involved.

Eight lots of approximately 400 poults each were started in duplicate from eight of these supply flocks. Poults from the ninth supply flock were started as a single lot of 184 poults. The tenth lot consisted of a pen of 200 males, and a pen of 200 females that were progeny of a flock classified as noninfected. These had been sexed immediately after the operator had sexed poults from an infected supply flock. The operator had been specifically instructed not to wash his hands before handling the experimental lot.

*Hatching and delivery of 1954 poults.* The day-old poults were delivered by each of three branch hatcheries to a central

TABLE I

Infectious sinusitis status of the 1954 commercial supply flocks which produced experimental poults

| Flock designation | Symptoms observed in supply flocks | | |
|---|---|---|---|
| | April* | By owner | June |
| A | None | None | None |
| B | None | None | None |
| C | None | None | None |
| D | None | None | None |
| E | None | In brooder house | No record |
| F | None | At about 6 months | None |
| G | None | of age | None |
| H | None | | Yes |
| I | Acute symptoms | Soon after start of egg production | No record |

* Observed by one of writers; other observations by owner or hatchery personnel.

point for toe-marking, banding, and reshipment to the brooding farm. Delivery dates and hatchery practices are shown in table 2. To minimize disease transmission at the assembly point, hatchery personnel were instructed 1) to handle progeny from the noninfected flocks first, 2) to wash their hands between lots, and 3) to separate lots to greatest extent possible. The poults came under the direct supervision of the writers upon delivery to the brooding farm where they remained until eight weeks old.

*Brooding of 1954 poults.* Brooding facilities were rented on a vacant commercial poultry farm. Figure 1 shows the arrangement of the five buildings used and assignment of poults to pens. Partitions in houses 1 and 2 allowed separation of lots, but the board and wire construction provided no isolation between lots. It was considered more important to determine whether poults from infected breeders would show

TABLE II

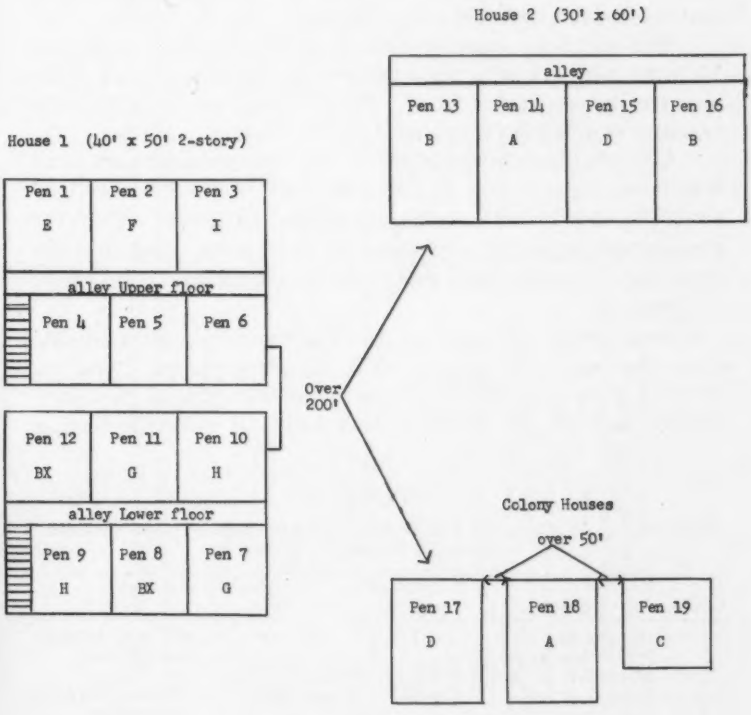
Delivery dates, hatchery source, and symptoms of infectious sinusitis in experimental poults (1954)

| Supply flock Designation | Sinusitis | Delivery date | Poults No. | Hatchery * | Age in days first symptoms noted** | |
|---|---|---|---|---|---|---|
| A | None | 5/31 | 400 | M | None | None |
| B | None | 5/27 | 400 | P | None | None |
| C | None | 5/27 | 184 | P | None | ----** |
| D | None | 5/31 | 400 | P | None | None |
| E | Infected | 5/27 | 400 | V | 26 | 30† |
| F | Infected | 5/27 | 400 | V | 28 | 28 |
| G | Infected | 5/27 | 400 | V | 27 | 27 |
| H | Infected | 5/31 | 400 | V | 1 | 1 |
| I | Acute | 5/31 | 400 | P | 28 | 28† |
| BX§ | None | 5/31 | 400 | P | 21 | 24 |

* Practices followed by each of three branch hatcheries:
M. All eggs set were from supply flocks classified as free of infectious sinusitis.
P. Eggs from breeders classified as free of infectious sinusitis were set and hatched in separate machines from eggs from infected flocks.
V. Each progeny lot was hatched separately.
** Each of duplicates shown separately; poults from supply C were all in one pen.
† Symptoms were not extensive in these lots until 8 weeks of age.
§ Sexed poults from supply flock B.

evidence of the disease than to ascertain which supply flock was involved in the transmission. The assignments made gave the best available isolation of progeny of noninfected from infected breeders. In the case of progeny from noninfected breeders brooded in house 2, the calculated risk that all these poults would not have an outbreak had to be taken.

Hover-type gas brooders were used. A commercial turkey starter ration was fed and the poults were cared for in a conventional manner by one caretaker.




A to I represent poults from supply flocks listed in table 2.

*Fig. 1.* Location and diagrammatic floor plans of buildings used in the 1954 brooding experiment showing distribution of poults.

*Perpetuation of infected and noninfected lines.* At the completion of the 1954 brooding period, the infected and noninfected stock were transported to separate, newly prepared ranges. These birds were to be maintained as separate breeding lines until the fourth generation reached market age. Breeders negative to the pullorum disease test were selected at about 24 weeks of age. Respiratory symptoms had spontaneously disappeared prior to that time. Swollen sinuses had been controlled by treatment with silver nitrate. The birds were vaccinated for fowl pox, erysipelas, and Newcastle disease at the time of selection.

The hatching seasons covered relatively short spans of 10 to 16 weeks, and in 1955 the season was interrupted when brooder houses were filled. Poults from each flock were brooded separately except as noted in table 4.

Criteria used to ascertain if egg transmission occurred were close observation of poults for symptoms of infectious sinusitis, and the serum plate agglutination test of Adler[1] for pleuropneumonia-like organisms (PPLO test). Bird inoculations and culture in suspected cases were used as a final check of infection.

*PPLO tests.* Antigen for the PPLO tests became available after the beginning of the 1955 breeding season. Sera remaining after the 1955 pullorum tests were frozen for later testing. In 1956, the breeders were tested at pullorum testing

TABLE III

Summary of serum plate PPLO agglutination tests of 1955 infected breeders at 10 months of age

| Pen No. | Treatment 15-21 weeks of age | Number tested | Results when read at 3 min. | 8 min. |
|---|---|---|---|---|
| 1 | 150 grams furazolidone/ton of mash* | 114 | all neg. | 27 neg., balance incomplete |
| 3 | None | 132 | all neg. | 27 neg., balance incomplete |
| 5 | 200 grams oxytetracycline/ton of mash | 114 | all neg. | 33 neg., balance incomplete |

* Pelleted mash and whole grains were fed free choice.

TABLE IV

Serum plate PPLO agglutination tests of the 1956 and 1957 experimental breeders

| Pen no. | Date hatched | Breeding line | No. started† (Males: females = 1:10) | Percentage of birds reacting to the test†† | | |
|---|---|---|---|---|---|---|
| | | | | 1956 Breeders | | |
| | | | | 24 weeks | 47 weeks | 54 to 60 weeks |
| 3 | 5/16/55 | infected | 137††† | 11.5 | 2.9 | No test |
| 1 | 3/14/55 | infected | 165 | 0.0 | 1.3 | 14.9 |
| A | 5/23/55 6/6/55 | noninfected | 151 | 0.0 | 4.7 | No test |
| 7 | 3/14/55 | noninfected | 165 | 0.0 | 0.0 | 1.3 |

| | | | | 1957 Breeders | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 24 weeks | 30 weeks* | 34 weeks | 38 weeks | 42 weeks |
| 7 | 1/9/56 | infected | 66** | 0.015 | 0.015 | 0.016 | 0.0 | no test |
| 1 | 3/12/56 | infected | 127 | 12.6 | 2.37 | 16.4 | 0.0 | 10.0*** |
| C | 5/14/56 | noninfected | 125 | 4.0 | 0.81 | 0.0 | 0.8 | 3.84 |
| A | 7/16/56 | noninfected | 102 | 4.0 | 1.96 | 3.2 | 1.1 | 3.2 |

† Noninfected line males used in all flocks to achieve uniformity in breeding between lines are included in figures of infected flocks. Noninfected line maintained as a closed flock.

†† All reactions were incomplete.

††† Excluding 1st test of 19 noninfected line females added to flock at 24 weeks of age.

* Lights were turned on when the breeders were approximately 30 weeks of age.

** Females in this pen were a mixture of infected and noninfected stock; a part of each brooded together; all were reared together since 8 weeks old.

***Ten random samples tested; all females.

time and as shown in table 4. The 1957 breeders were tested at pullorum testing time, at time of lighting at approximately 30 weeks of age, and every four weeks thereafter as listed in table 4.

Ten random sera samples were routinely taken from all 1957 progeny at 7 or 8 weeks of age and subjected to the PPLO test. In a few hatches, the tests were run at ages up to 25 weeks in addition to, or in lieu of, the routine procedure. All tests were performed by the same individual.

Additional details and deviations from routine procedure will be indicated in pertinent sections that follow.

## RESULTS

*1954 brooding experiment.* As shown in table 2, all poults from infected breeders showed symptoms suggestive of infectious sinusitis before one month of age. At one month of age, symptomatic evidence of the disease was characteristic and extensive. However, tables 1 and 2 show that the supply flock that had an outbreak in the brooder house and the flock with the acute infection had produced progeny that did not show marked symptoms until they were eight weeks of age. Progeny of one of the three supply flocks that had infectious sinusitis at about six months of age had an immediate serious outbreak. The supply flock involved was flock H in table 1 and it was reported to have symptoms of the disease after delivery of the poults. Thirteen poults from these breeders were dead when delivered; these showed excess tracheal mucus and air sac lesions. During the first week, 51 poults died in one pen and 56 died in the other. Gasping was evident from the start and at *six* days of age poults in both pens showed symptoms characteristic of infectious sinusitis. Other infections were ruled out by appropriate bacteriological procedure.

The progeny of noninfected breeders that had been sexed after sexing progeny of an infected flock also showed symptoms of the disease. It is not clear whether infection in this case resulted from the sexing operation or from possible cross infection because of proximity to infected poults. Duplicate lots always tended to react similarly. Transmission of the dis-

ease to normal turkeys by sinus inoculation of sinus exudate confirmed the diagnosis of infectious sinusitis.

All poults from breeders classified as noninfected remained free of the disease. However, caseous lesions were found in the air sacs of a number of poults examined from the noninfected breeders. Cultural procedures and bird inoculations used failed to reveal the cause of these lesions.

*Perpetuation of infected and noninfected lines.* Egg transmission in poults hatched in 1955 from the infected line breeders was limited to a few individual poults in 6 of 11 hatches made from the infected flocks. No spread within any hatch was noted. In fact, close observation was required to detect the swollen sinuses, which was the usual manifestation. In some cases the swollen sinuses were first noted after the birds had gone to range at 8 weeks of age. Pleuropneumonia-like organs (PPLO) were isolated from the trachea in two instances, but the cultures were difficult to maintain even though isolates from other sources grew readily in the same media. Transmission of the disease by use of sinus exudate was possible. However, exudate from a male with a swollen sinus, typical of infectious sinusitis in all respects, produced no symptoms when injected into sinuses of test birds, despite repeated attempts. An observation of interest was that one female with swollen sinus penned with this male developed a cough characteristic of infectious sinusitis after reaching breeder age.

In the 1956 progeny, no clear-cut evidence of infectious sinusitis was observed, but the project was jeopardized by an outbreak of what was considered to be bluecomb as described by Pomeroy and Sieburth.[9] The poults of the second and third hatches of the season suffered losses in excess of 90 percent. Bluecomb with diminishing virulence continued to appear in succeeding hatches, except in three hatches which were run through two particular brooder houses where no evidence of the disease was noted.

One-half of the 1957 breeding flocks were selected from poults brooded in the houses which had shown no evidence of

bluecomb infection. All 1957 breeders and their progeny were free from clinical evidence of infectious sinusitis and no further bluecomb outbreaks occurred. Random PPLO tests routinely conducted on all 1957 progeny at 7 or 8 weeks of age and at a later age or only at a later age up to 25 weeks were negative.

The noninfected line concurrently maintained has remained free of clinical evidence of infectious sinusitis. Poults hatched for this experiment numbered 5440 in 1955 and slightly over 7500 each year for 1956 and 1957. The number of noninfected and infected poults was approximately equal each year. In addition to bluecomb, predators, blackhead, and brooderhouse coccidiosis were serious problems until controlled by appropriate measures.

*PPLO tests.* The sera of 1955 breeders that were frozen were found to be unsatisfactory for PPLO tests, because all birds, infected and noninfected alike, showed positive reactions. That this was due to freezing of the sera was confirmed by retest of the noninfected breeders when they were 10 months old. Also, when fresh sera negative to the test were frozen, thawed, and retested with the same antigen, reactions occurred in many sera so treated. When tested at 10 months of age, negative titers were encountered in infected flocks if the reading was made in three minutes after mixing serum and antigen. By delaying the reading to eight minutes or longer, incomplete reactions were encountered. A summary of tests of the 1955 breeders appears in table 3.

The results of tests conducted on the 1956 and 1957 breeders are shown in table 4. Reactions encountered were again of the incomplete type. In 1956 both of the noninfected line flocks were negative on the initial test. One of the infected line flocks was also negative on the initial test. In 1957 none of the flocks were free of some reaction on the initial test. In each year, one of the infected line flocks had an incidence of approximately 12 percent incomplete reactions at 24 weeks of age. The 1957 infected line flocks (pen 1) with 12.6 percent incomplete reactions in the first tests gave inconsistent results

in subsequent tests and fell to zero in the test 8 weeks after lighting. All flocks showed a low percentage of reactions at that particular period.

Only one 1957 flock in table 4 (pen 7) was free of reactions on the final test. The final test on this flock was taken 8 weeks after lighting and coincided with the low incidence found in all flocks at that time. It was noted that different birds accounted for reactions from test to test. This would indicate fleeting reactions, which may have been non-specific, in many individuals rather than persistent reactions in fewer individuals. These results are recorded without interpretation at this time, except to point out that the progeny of the 1956 and 1957 flocks were clinically free of evidence of infectious sinusitis.

Semen collected at the time of the last test from all males in 1956 pens A and 3 were cultured for PPLO by Dr. R. D. Conrad at the State College of Washington, Pullman and by Dr. Don Zander, Heisdorf and Nelson Farm, Kirkland, Washington. Both reported negative results.

## DISCUSSION

*1954 brooding experiment.* In the hatchery organization from which the experimental poults were obtained, infectious sinusitis had been encountered for several years. It was not widespread and did not attract undue notice until 1954 when a problem of infectious sinusitis in poults delivered by the hatchery organization was recognized. This history suggests a build-up of the infection over several seasons.

All infected breeders, from which poults were started in the 1954 brooding experiment, had received one or several courses of treatment with one or more of the antibiotics then commonly used for this purpose. These antibiotics were administered in the feed or water and by intramuscular injection or by injection alone. In one case, males received 500 mg or more of streptomycin by intramuscular injection.

The course of the disease in supply flock H listed in table 1 differs from its course in other infected flocks in that coughing was present at the end of the hatching season. Available

data do not satisfactorily explain this difference. It is possible that the coughing, observed clinically, was due to some cause other than infectious sinusitis. Progeny of this flock showed respiratory difficulty and lesions at one day of age and had an outbreak of infectious sinusitis during the first week.

In classifying flocks as free of infectious sinusitis, great dependence on the honesty and observation of the grower or hatchery representative for accurate history was necessary. Hence, it would not have been surprising to find that some flocks had been incorrectly classified as free of infectious sinusitis. However, no infected progeny resulted from supply flocks classified as noninfected in the field survey. Conversely, previous field experiences had not led to the expectation that all progeny of infected breeders would show evidence of the disease. Cross-infection between lots may explain this observation but this could not be definitely ascertained.

*Perpetuation of infected and noninfected lines.* Initial concern in this endeavor was to preserve the noninfected line free of infectious sinusitis. No difficulty was encountered in doing so on the basis of clinical evidence. The only evidence of possible infection in this line was the increase of incomplete reactions in the PPLO tests of breeder flocks in 1957. Progeny of this and all other 1957 flocks showed no clinical evidence of the disease and were negative on the routine PPLO test at 7 to 25 weeks of age.

Perpetuation of a naturally infected line by egg transmission was more difficult than was anticipated and resulted in apparent loss of the infection. Similar occurrences are not unusual in the field where it is commonly observed that poults with no clinical evidence of infectious sinusitis have been produced from infected flocks. Such observations have understandably bred skepticism that infectious sinusitis is egg transmitted. Scientific workers are no exception, *viz.*, the report of Grumbles and Boney.[3] However, evidence that egg transmission of infectious sinusitis does occur appears indisputable. Obviously, it does not occur in all progeny nor in all hatches of infected breeders. Van Roekel *et al.*[11] have inde-

pendently reported a similar finding in chronic respiratory disease (CRD), a PPLO infection of chickens. They state: "It is significant that CRD-free progeny can be raised from infected dams."

The observed minimal egg transmission in 1955 in the immediate progeny of the original infected stock could have been a potential source of perpetuation of the infection. Circumstances in these experiments did not allow a buildup from this source, but in the case of the hatchery organization concerned in the 1954 experiment, a serious egg transmission problem was encountered. The possible effect of mortality from intercurrent causes experienced in some hatches on egg transmission of infectious sinusitis is a conjecture. The possible effect of the interrupted hatching in 1955, the relatively short hatching seasons, and of using males from the noninfected line in 1956 and 1957 likewise are not known. It might be added here that it was felt that the introduction of birds from the noninfected, and presumably susceptible, line to the infected line would enhance spread of the disease as well as accomplish the prime purpose of effecting more uniform breeding in the two lines. The observation period of the breeding lines covered five generations including the original field flocks. Three generations of breeders and four of progeny were under the direct supervision of the writers.

The need for experimentation, with the purpose of ascertaining a possible pattern of egg transmission is emphasized by the results reported herein. This is essential to an understanding of egg transmission and to effect practical measures to control egg transmission of infectious sinusitis.

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SUMMARY

Poults from four supply flocks of a commercial hatchery organization with no clinical evidence or history of infectious sinusitis that were hatched and reared in isolation remained clinically free of the disease. Poults from five infected supply flocks and poults housed with them suffered an outbreak of the disease during the brooding period.

The noninfected stock, maintained yearly as supply flocks for experimental poults, has shown no clinical evidence of the disease. A total of five generations including the original field flocks were observed. Concurrent perpetuation of the infected stock under similar conditions resulted in the production of progeny that showed no clinical evidence of the disease in the last two generations.

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# DETECTION OF BACTERIAL CONTAMINANTS IN LIVE VIRUS POULTRY VACCINES*

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OVER 2 billion doses of live virus poultry vaccines are produced annually. Embryonated eggs used in manufacture of these vaccines, when contaminated, can be a means of contaminating vaccines. Although Federal regulations[5,6] exist for testing of these vaccines they do not appear adequate. Johnson *et al.*[4] and others[1,7] have brought out the importance and need for additional testing standards. Such standards should have as their goal the eventual establishment of Federal regulations requiring sterility of poultry vaccines.

Membranes of cellulose derivatives for bacterial analyses were introduced into the United States by Goetz[2] and are now used widely in water and dairy sanitation. Holdowsky,[3] applied the membrane filter technic to sterility testing of antibiotic preparations. This technic with certain modifications was investigated for its applicability and compared to the agar plate method for detecting bacterial contaminants in experimental and commercial live virus poultry vaccines.

Little work has been done to determine if contaminated vaccines exist industry wide; whether antibiotics added during preparation of the vaccines completely eliminate bacterial

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contaminants; and if not, how these contaminating bacteria can be detected in antibiotic-treated vaccines. This prompted a study to evaluate methods for the isolation of bacterial contaminants from such vaccines.

## MATERIALS AND METHODS

Experimental vaccines were prepared so that 1 ml represented one-third sterile chorio-allantoic fluids, one-third antibiotic mixture in sterile 0.85 percent saline (1000 units of penicillin and 5 mg of streptomycin), and one-third saline suspension of washed bacterial cells.

Two series of experimental poultry vaccine were prepared. In one series no attempt was made to dilute the bacterial cells of an 18 to 24 hour trypticase soy broth culture which were twice washed and resuspended to their original volume. In the second series, 10-fold dilutions of the twice washed 18 to 24 hour trypticase soy broth cultures were made and certain dilutions were selected for use.

Vaccines with sterile saline replacing the antibiotic mixture were prepared as controls. Evidence of growth and number of colonies recovered from the control vaccines served as the standard of bacterial contamination for comparative purposes. Each sample of experimental vaccine was artificially contaminated with one of the following bacterial types: *Salmonella pullorum, Salmonella gallinarum, Salmonella typhimurium, Pasteurella multocida, Escherichia coli, Aerobacter aerogenes, Alcaligenes fecalis, Streptococcus fecalis, Staphylococcus aureus, Proteus vulgaris, Paracolobactrum* sp., and *Bacillus subtilis.*

Fifty-three different lots of commercial Newcastle disease vaccine supplied by 18 licensed manufacturers were available for testing.

The filtering apparatus consisted of basic parts manufactured by the Millipore Company, Watertown, Massachusetts. It included a glass filter holder, glass receptacle, metal tension clamp, membrane filters, and a vacuum flask (1 liter) with a cotton plug inserted in the side arm. The membrane

filters prepackaged for sterilization had a diameter of 47 mm and were of the hydrosol assay type. The calculated pore size is 0.45 u ± 0.02 u.

Pieces of equipment separately wrapped were autoclaved at 121 C for 15 minutes under 15 pounds pressure. After cooling, the apparatus was assembled in a continuous operation by inserting the filter holder in the mouth of the vacuum flask; placing a sterile filter on the filter holder with the aid of smooth tipped curved forceps; and placing the glass receptacle over the top of the filter holder. The metal clamp was applied to the union of the filter holder and receptacle thus enclosing the membrane filter. The top of the glass receptacle remained covered except at times when fluids to be filtered were poured into the receptacle. Sterile tubing connected the vacuum flask to a vacuum line.

One hundred doses of commercial vaccine or 1 ml of experimental vaccine were added to 100 ml of sterile distilled water. The mixture was poured into the glass receptacle through an opening formed by raising one side of the sterile covering over the top of the glass receptacle. Gentle vacuum was applied to facilitate the filtering action. Three separate aliquots, 250 ml each, of sterile distilled water were then passed through the filter in an identical manner. The metal clamp and glass receptacle were removed exposing the filter. The filter was grasped at its edge with sterilized smooth tipped straight forceps and transferred to the hardened surface of trypticase soy agar plates. The free edge of the filter was first touched to the surface of the agar and with a rolling action the remainder of the filter was lowered to the surface thus avoiding entrapment of air between the filter and agar. The plates were incubated at 35 C for 48 hours and then examined for evidence of bacterial growth. Detection of bacterial colonies was greatly facilitated by the use of a Quebec colony counter.

Sufficient quantities of experimental vaccines were prepared to permit filtering 15 and 60 minutes after preparation to detect any variation in inhibitory action of the antibiotics

resulting from the length of exposure.

A second method of testing experimental and commercial vaccines was used. One ml of experimental vaccine or 100 doses of commercial vaccine was mixed with 9 ml of fluid trypticase soy agar and the mixture poured into sterile petri dishes and incubated at 35 C for 48 hours. This method is hereinafter referred to as the "conventional" method.

## RESULTS

*Experimental vaccines.* Bacterial contaminants artificially added to simulated poultry vaccines could not be detected by the conventional method of testing due to the inhibitory action of antibiotics (tables 1 and 2). In most instances it was possible to recover bacteria from vaccines when tested by the membrane filtration method irrespective of whether undiluted cultures (table 1) or diluted cultures (table 2) were used.

In four instances (*Escherichia coli, Streptococcus fecalis, Alcaligenes fecalis,* and *Bacillus subtilis*), the antibiotics were completely inhibitory (table 1).

*Pasteurella multocida* recovered after a contact period of 60 minutes with the antibiotics showed aberrant colonial morphology, probably as a result of the action of antibiotics. Similar findings were encountered less often with the Salmonella species studied.

Bacterial growth in some instances was detected by the membrane filtration method in the antibiotic-treated simulated vaccines contaminated with limited numbers of bacteria (table 2). *Salmonella gallinarum* and *Pasteurella multocida* were recovered from the samples after 15 and 60 minutes of contact with antibiotics. *Samonella pullorum* and *Salmonella typhimurium* were recovered after a 15 minute period of contact but not after 60 minutes. Growth of *Escherichia coli* could not be detected after 15 or 60 minutes of contact with antibiotics.

*Commercial Newcastle disease vaccine.* The membrane filtration and conventional methods of testing were applied to samples of commercial vaccine previously released for

TABLE I

Bacterial growth recovered from artificially contaminated (undiluted cultures), antibiotic-treated vaccines by membrane filtration and conventional methods

| Organism | Membrane filtration method | | | | Conventional method | | | |
|---|---|---|---|---|---|---|---|---|
| | Vaccine with antibiotics | | Control vaccine no antibiotics | | Vaccine with antibiotics | | Control vaccine no antibiotics | |
| | 15 min* | 60 min* | 15 min | 60 min | 15 min* | 60 min* | 15 min | 60 min |
| *Salmonella pullorum* | + | + | + | + | — | — | + | + |
| *Salmonella gallinarum* | + | — | + | + | — | — | + | + |
| *Salmonella typhimurium* | + | + | + | + | — | — | + | + |
| *Pasteurella multocida* | + | + | + | + | — | — | + | + |
| *Eschericha coli* | — | — | + | + | — | — | + | + |
| *Aerobacter aerogenes* | + | — | + | + | — | — | + | + |
| *Streptococcus fecalis* | — | — | + | + | — | — | + | + |
| *Alcaligenes fecalis* | — | — | + | + | — | — | + | + |
| *Staphylococcus aureus* | + | + | + | + | — | — | + | + |
| *Proteus vulgaris* | + | + | + | + | — | — | + | + |
| *Paracolobactrum* | + | + | + | + | — | — | + | + |
| *Bacillus subtilis* | — | — | + | + | — | — | + | + |

* Time intervals indicate period of exposure to antibiotics before testing
+ Bacterial growth
— No bacterial growth

TABLE II

Number of bacterial colonies recovered from artificially contaminated (diluted cultures), antibiotic-treated vaccines by membrane filtration and conventional methods

| Organism | Dilution | Membrane filtration method | | | | Conventional method | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Vaccine with antibiotics | | Control vaccine no antibiotics | | Vaccine with antibiotics | | Control vaccine no antibiotics | |
| | | 15 min* | 60 min* | 15 min | 60 min | 15 min* | 60 min* | 15 min | 60 min |
| *Salmonella pullorum* | $10^7$ | 7** | — | 42 | 40 | — | — | 50 | 58 |
| *Salmonellla typhimurium* | $10^8$ | 33 | — | 99 | 24 | — | — | 33 | 15 |
| *Salmonella gallinarum* | $10^5$ | 46 | CG | 51 | 49 | — | — | no | sample |
| *Pasteurella multocida* | $10^5$ | 32 | CG &AG | TNTC | TNTC | — | — | TNTC | TNTC |
| *Escherichia coli* | $10^7$ | — | — | 104 | 109 | — | — | no | sample |

* Time intervals indicate period of exposure to antibiotics before testing
** Number of colonies detected
CG Confluent growth
AG Aberrant growth
TNTC Too numerous to count
— No bacterial growth

market. The findings of bacterial contamination in these vaccines are shown in table 3.

Control samples prepared without antibiotics for serial lots 1, 2, 4, 9, 12, 16, 23, and 28 were available for comparative studies. The findings for these samples, when tested by the membrane filtration method, were similar to those obtained on identical samples of marketable vaccine containing antibiotics. Of particular interest was the testing of serial lot 28. This vaccine was grossly contaminated and it was necessary to reduce the inoculum to 0.1 dose in order to arrive at countable colonies on the filter.

TABLE III

Recovery of bacterial contaminants from commercial live virus poultry vaccines by membrane filtration and conventional methods

| Source* | Serial** lots | Number of colonies per 100 doses | |
|---|---|---|---|
| | | Membrane filtration method | Conventional method |
| A | 1 | — | — |
| B | 2 | — | — |
| | 3 | 77 | 10 |
| C | 4 | 3 | — |
| | 5 | 1 | — |
| | 6 | 49 | — |
| | 7 | 2 | — |
| | 8 | 22 | — |
| D | 9 | SG | 5 |
| | 10 | 27 | 10 |
| | 11 | 72 | — |
| E | 12 | 1 | 1 |
| | 13 | 6 | — |
| | 14 | 2 | — |
| | 15 | 3 | — |
| F | 16 | 18 | 7 |
| | 17 | 93 | — |
| | 18 | TNTC | — |
| | 19 | 3 | — |
| | 20 | — | — |
| G | 21 | — | — |
| | 22 | 2 | — |
| H | 23 | — | — |
| | 24 | — | — |
| | 25 | -- | — |
| | 26 | — | — |
| | 27 | — | — |

TABLE III—Continued

| Source* | Serial** lots | Number of colonies per 100 doses: Membrane filtration method | Number of colonies per 100 doses: Conventional method |
|---|---|---|---|
| I | 28 | 5000 | — |
| | 29 | — | — |
| | 30 | — | — |
| | 31 | 1 | — |
| | 32 | — | — |
| | 33 | — | — |
| J | 34 | 304,000 | 430,000 |
| | 35 | 61 | — |
| | 36 | TNTC | — |
| | 37 | 83 | — |
| | 38 | 44 | — |
| K | 39 | 140,000 | 150,000 |
| L | 40 | 18 | — |
| | 41 | — | — |
| | 42 | — | — |
| M | 43 | 423 | 158 |
| | 44 | 180 | 9 |
| N | 45 | TNTC | 4 |
| O | 46 | TNTC | TNTC |
| | 47 | — | — |
| | 48 | — | — |
| P | 49 | TNTC | — |
| | 50 | TNTC | — |
| | 51 | — | — |
| Q | 52 | 42 | — |
| R | 53 | SG | — |

TNTC Too numerous to count
SG Spreading growth
— No bacterial growth
* Letter refers to manufacturers
** No's. 1-53 refer to different lots of vaccines

Table 3 includes two manufacturers, J and K, who at one time did not add antibiotics during processing of their vaccines. Serial lots 34 and 39 were produced without the addition of antibiotics. These two vaccines were grossly contaminated and it was necessary to reduce the inoculum to 0.01 dose for testing purposes. The results indicated close correlation of the two methods of testing vaccines when antibiotics were absent.

Of the 53 different lots of vaccine tested (table 3), 35

were contaminated when tested by the membrane filtration method, 11 of which contained more than 100 colonies per 100 doses of vaccine. No attempt was made to identify all contaminants but Gram stains revealed the presence of Gram positive cocci, Gram negative, and Gram positive bacilli.

## DISCUSSION

By the membrane filtration method, bacterial cells were retained on the filter and washed free of the residual antibiotic by subsequent washings with sterile distilled water, affording those bacteria still viable an opportunity to grow and multiply when the filter was placed on trypticase soy agar.

Bacterial contamination in experimental vaccines and in many serial lots of the commercial vaccines was not detected by the conventional method of testing; whereas, bacteria were detected by the membrane filtration method indicating its usefulness in nullifying the inhibitory action of antibiotics in the vaccines so that a more valid assay for bacterial contaminants could be made.

The membrane filter has a disadvantage in that vaccines with heavy particulate matter will not pass the filter. Vaccines prepared from chorioallantoic fluids were tested without difficulty. In some instances when vaccine samples will not pass the filter, it is possible to reduce the amount of vaccine for filtering; for example, 50 doses instead of 100 doses. Separate filtering runs can be made and the number of colonies on each filter added to arrive at the number of colonies per 100 dose sample.

Vaccines grossly contaminated such as 18, 28, 34, 36, 39, 43, 44, 45, 46, 49, and 50 (table 3) were considered unsuitable for marketing. Such vaccines demonstrated carelessness in vaccine manufacturing and a misrepresentation of the vaccine to the user.

Licensed live virus poultry vaccines available on the market are contaminated with various types of bacteria and many serial lots are grossly contaminated. The addition of antibiotics to vaccines does have an advantage in reducing the number of bacterial contaminants in embryo material harvest-

ed for vaccine production, but there are indications that antibiotics are being added for the purpose of taking short cuts in production and replacing sound laboratory practices of sterile technics. To assure that grossly contaminated embryo material is not used for vaccine production, this material should be tested for sterility before the addition of antibiotics.

## SUMMARY

Two methods of testing were described and compared as to their effectiveness in detecting bacterial contaminants in antibiotic-treated live virus poultry vaccines. The results indicated that the conventional method of testing did not detect all bacterial contaminants. The use of this method was unsatisfactory for bacterial examination of vaccines containing antibiotics. The membrane filtration method was a useful technic for detecting bacterial contaminants in such vaccines and its use is to be recommended.

The results of testing commercial lots of Newcastle disease vaccine indicated that licensed manufacturers need to be more careful in the selection of embryonic fluids and tissues for vaccine production. To assure this, a requirement for the bacterial examination of this material before the addition of antibiotics is highly recommended.

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# EXPERIMENTAL ORNITHOSIS IN TURKEYS*

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ORNITHOSIS, an infection of non-psittacine birds, is caused by members of the psittacosis-lymphogranuloma-venereum (PLV) group of disease agents. Investigation of this disease in turkeys began in 1948 after a number of cases of psittacosis occurred among employees of a Texas poultry processing plant following the handling of diseased turkeys.[3] The investigation revealed ornithosis epizootics among certain flocks of turkeys in that state. Ornithosis agents were later isolated from infected turkeys and one isolate was characterized as a highly toxic, large elementary body, obligately intracellular organism belonging serologically to the PLV group.[1,4] Subsequent proven outbreaks of ornithosis in turkeys in Texas in 1952, New Jersey, Texas and California in 1954, and in Oregon, Minnesota and California in 1956 have shown that the disease has become a potentially serious threat to the turkey industry as well as a hazard to persons handling affected birds.

Studies by the author on experimental ornithosis in turkeys began in late 1954 following isolations of ornithosis agents from turkey tissues shipped from areas of suspected outbreak in New Jersey and California. Earlier investigations by Pate, Boney and Delaplane[5] had characterized the

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gross pathology of experimentally and naturally infected turkeys, but further studies were desirable to clarify the route of the disease agent in birds infected by the airborne and oral routes.

## MATERIALS AND METHODS

Broad-breasted bronze turkeys, 10 to 23 weeks of age, were obtained from various California sources for test purposes. The birds were housed in concrete-floored cubicles measuring 3½ x 14 x 8 feet separted by wire mesh and continuously exposed to the cool, humid climate and generally westerly winds of San Francisco. They were fed a mixture of turkey mash and chick starter feed (without antibiotics).

The ornithosis agent used in all the experiments and referred to as the NJ1 isolate was recovered from the muscle of an acutely diseased turkey obtained from a 1954 outbreak of ornithosis in New Jersey. White, male, Princeton mice, 3 to 5 weeks of age, were used for isolation or titration of ornithosis infectious particles. The NJ1 isolate was uniformly fatal to mice when injected by the intraperitoneal, intranasal or intracerebral routes. It was irregularly fatal when given intraperitoneally to guinea pigs or rice birds and innocuous to pigeons by the intracerebral route. Smears of infected yolk sac or mouse peritoneal exudate stained by Macchiavello's method usually showed numerous intracellular elementary bodies. Titration of numbers of infectious particles per gram of tissue was performed by injecting 0.5 ml of decimal dilutions of a 20 percent tissue suspension in beef heart broth into mice by the intraperitoneal route with 5 mice per dilution. Death within 21 days after inoculation was the endpoint and upon termination of the test, all mice were examined for lesions of ornithosis. Fifty percent endpoints were calculated by the method of Reed and Muench.[6]

Test inocula consisted of infected yolk sac or mouse spleens and livers ground and suspended in beef heart broth. Aerogenic infections were obtained by exposing birds to an infective mist of approximately one micron particle size produced by an impinger-type aerosol device. Oral infections were

produced by inserting gelatine capsules containing suspensions of infective material into the turkey esophagus. Intraperitoneal inoculations were made by syringe and needle.

## RESULTS

Preliminary tests employing the intratracheal and intraperitoneal routes with large doses of egg-propagated infectious particles produced acute respiratory and systemic disease in most birds with 40 percent mortality. Within 2 to 4 days, affected birds were depressed in appearance, usually showed a marked hyperthemia and anorexia, and excreted yellowish-green gelatinous droppings containing ornithosis agents (figure 1). Death due to disease occurred within 8 to 15 days after inoculation. Indirect complement fixation (ICF) titers rose rapidly after the 7th day to as high as 1:2048 on the 29th day and were maintained at relatively high levels for at least 3 months. Necropsy of birds dying in the tests showed gross lesions typical of ornithosis (figure 2) as decribed by Pate, *et al.*[5] and later by Davis.[2] Ornithosis organisms were isolated from all tissues tested of birds dying of the disease and could be recovered from the cloacal contents of surviving birds up to 27 days after inoculation. In the first two tests, however, infectious particles could not be recovered from birds sacrificed 44 or more days after exposure.

Early in each test, normal poults were placed in the cages with infected birds as susceptible contact controls. Of five contacts in the preliminary tests, all became infected as evidenced by rise in ICF titer except one which died of acute ornithosis before responding serologically to the disease. Ornithosis agents were recovered from seven separate tissues of that bird by mouse passage tests.

Studies were then undertaken to trace the path of the ornithosis agent through the turkey after exposure to the NJ1 isolate by the air-borne or oral route. These two routes were chosen as the most likely routes of infection in natural outbreaks. Since it was known that the birds excreted infectious particles in their droppings at frequent intervals when

*Fig. 1.* Depressed appearance of turkeys ten days after exposure to New Jersey 1 strain of turkey ornithosis virus. Droppings at this acute stage of the disease are yellowish-green and gelatinous.

experiencing acute ornithosis, it was assumed that the primary vehicle for passage of infective material from one bird in a flock to another was the excreta. To account for high morbidity rates in flock infections, it was conceivable that the transfer of infection could best occur via the respiratory tract by exposure to aerosols of dried excrement in dusty, enclosed areas. Entry of the organism through the gastrointestinal tract as a result of coprophagous activity was considered another possibility since natural outbreaks have occurred in

*Fig. 2.* Gross lesions of a turkey infected by the intraperitoneal route with the New Jersey 1 strain of turkey ornithosis virus. Evidence of severe pericarditis, airsaculitis and hepatomegaly is present. Arrow points to heavy fibrinous deposits on abdominal airsac.

## TABLE I

Quantitative distribution of the ornithosis agent in tissues

| Hours after infection | Log Mouse $LD_{50}$ Per Gram Tissue or Per Milliliter Fluid | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tur | Lu | AS | PCS | PW | B | L | S | K | BM | T. or OV. | Mu | CC | CF |
| 4 | Neg. | 1.00 | 1.30 | 1.30 | Neg. | Neg. | Neg. | Neg. | | | | | | |
| | Neg. | 1.50 | 2.30 | 1.30 | 1.00 | Neg. | Neg. | 1.62 | | | | | | |
| 8 | Neg. | 1.50 | 1.63 | Neg. | 1.86 | Neg. | Neg. | Neg. | | | | | | |
| | Neg. | 1.00 | 1.80 | Neg. | 0.74 | Neg. | Neg. | Neg. | | | | | | |
| 24 | 3.50 | 8.0 | 8.0 | 1.80 | 3.50 | Neg. | Neg. | 1.50 | | | | | | |
| | 3.50 | 8.0 | 5.5 | 1.80 | 3.67 | Neg. | Neg. | 1.50 | | | | | | |
| 36 | | 8.0 | 8.0 | 3.30 | 5.5 | Neg. | 3.33 | 3.00 | 1.30 | | | | | |
| | | 8.0 | 8.0 | 3.30 | 5.5 | Neg. | 2.23 | 3.00 | 1.5 | | | | | |
| 48 | 6.00 | 8.0 | 5.05 | 4.83 | | 0.33 | Neg. | 4.00 | 2.60 | | | | | |
| | 5.26 | 8.0 | 8.0 | 5.30 | | Neg. | Neg. | 3.50 | 3.00 | | | | | |
| 72 | | 6.52 | 6.00 | 5.23 | | 0.50 | 3.37 | 4.00 | 2.23 | | | | Neg. | |
| | | 5.61 | 6.52 | 6.33 | | 0.44 | 2.23 | 5.33 | 2.00 | 1.00 | 1.00 | | 1.00 | |
| 96 | 3.76 | | 7.00 | 6.00 | | 2.00 | 3.00 | 6.00 | 4.23 | 1.23 | 3.23 | | 1.93 | |
| | | 7.67 | 7.00 | 6.50 | | 1.23 | 3.50 | 5.00 | 4.33 | 1.76 | | | 2.60 | 3.23 |
| 120 | | 8.33 | 7.23 | 5.67 | | 3.37 | 3.67 | | | | | 2.23 | Neg. | |
| | | 7.50 | 7.23 | 6.50 | | 3.30 | 4.50 | | | | | 2.76 | 2.80 | 1.00 |
| 14 days Death | | 5.50 | 5.33 | 6.67 | | 1.60 | 4.67 | 4.67 | | | | 2.23 | 5.13 | |
| 12 Days | | 8.0 | | 7.85 | | | 7.33 | 8.0 | 7.40 | 5.5 | 7.0 | | | |

Note: Underlining a number (e.g. 8.0) indicates the titration was not carried to the end point, and the figure is an estimate based on average day to death data from complete titrations.
Tur = Turbinates; Lu = Lung; AS = Airsacs; PCS = Pericardial sac; PW = Peritoneal Washing; B = Blood; L = Liver; S = Spleen; K = Kidney; BM = Bone Marrow; T. or Ov. = Testes or Ovary; Mu = Muscle; CC = Cloacal Content; CF = Crop Fluid.

areas where dust aerosols were of a minimal order.

*Infection by the air-borne route.* An airborne-infection trial on 6 turkeys produced acute systemic disease in 100 percent of exposed birds as evidenced by daily isolations of the disease agent from the blood stream from the 2nd to the 9th day and the rapid rise in ICF titer.

In the principal airborne route experiment (table 1, figures 3, 5) 20 poults, 14 weeks of age, were exposed for 4 minutes to an aerosol containing 40,000 mouse $LD_{50}$ of the NJ1 isolate prepared from a 10 percent homogenate of infected mouse spleens and livers. Two birds were killed by exsanguination at each of 4, 8, 24, 36, 72, 96 and 120 hour intervals after exposure to the infective mist. At necropsy each bird was examined for gross lesions and the following tissues were removed aseptically to determine the number of mouse $LD_{50}$ per gram tissue:

| Tissues titrated every time interval | Tissues titrated at selected time intervals |
|---|---|
| Lung | Turbinates |
| Abdominal and thoracic air sacs | Bone marrow |
| Pericardial membrane | Testes or ovary |
| Blood | Kidney |
| Liver | Muscle |
| Spleen | Cloacal content |
| | Crop fluid |

The data indicated that infective virus in the aerosol reached the air cells of the lung and the air sacs where it multiplied and achieved a very high concentration in 24 hours. The agent was in the pericardial membrane and on the mesentery within 4 hours implying that very shortly after infection virus particles were introduced into tissues and membranes contiguous to the air sac system. Within 48 hours, organisms were detectable in the blood. From the rapid increase in number of infective particles in the lung and air sacs, it was evident that these tissues were primary foci for multiplication. The data would indicate that the pericardial

*Fig. 3.* Gross lesions of turkey exposed to aerosol of NJ1 virus. The bird died seven days after infection. The heart is enlarged and is covered with thickened and injected pericardial membrane. Hepatomegaly is present along with rupture of hepatic vein.

sac was a site of less rapid multiplication. After detectable blood stream invasion, the virus could be found throughout the body—in the kidney, reproductive organs, muscle and bone marrow. Transudates containing large numbers of in-

fective particles collected in volume in the thoracic cavity. Cloacal material contained elementary bodies within 72 hours after infection indicating that at that time the birds began to excrete the agent.

On the second day after exposure, fibrin plaques appeared

*Fig. 4.* Gross lesions of turkey after exposure to NJI ornithosis virus by contact. Close view shows severe pericarditis and hepatomegaly. Note the close similiarity of these lesions to those in Fig. 3.

on the heart. In four days, the entire epicardium was covered with a heavy layer of fibrin which later formed a thick crust (figure 4).

*Oral route infection tests.* In a preliminary test in which eight turkeys were exposed to 340,000 mouse $LD_{50}$ by the

gastro-intestinal tract, no clinical signs of disease were noted for almost 7 weeks. At the fourth week, these birds and 4 contact control turkeys were accidentally exposed to heavily infected turkeys in an adjoining cage separated by wire mesh fence. Within 24 days after this unscheduled exposure, four turkeys including two contact control birds, died of acute ornithosis, two of them with no ICF titers, indicating death followed soon after infection.

In another experiment designed to follow the path of the virus after oral feeding, 20 turkeys, 23 weeks of age, were infected by inserting a gelatine capsule containing 40,000 mouse $LD_{50}$ of the NJ1 isolate from infected mouse spleen and liver homogenate, into the esophagus. Five normal turkeys were placed in the turkey runways with the infected birds to serve as contact controls. Two test birds were sacrificed at intervals of 1, 2 and 5 days after exposure. The tissues listed below were removed and titrated in mice to determine the number of infective particles present in the tissue:

Tissues examined in Oral Infection Experiment

| | |
|---|---|
| Esophageal washing | Cecal contents |
| Crop contents | Colon contents |
| Crop wall | Liver |
| Proventriculus wall | Spleen |
| and contents | Kidney |
| Gizzard contents | Mesentery |
| Duodenum contents and wall | Air sacs |
| Ileum contents | Blood |

The remaining birds including the controls were bled at approximately 10 day intervals for serum specimens and for blood to subinoculate into mice. The sera were subjected to the indirect complement fixation test.

Gross examination of turkeys sacrificed at 1, 2 and 5 days after inoculation indicated no apparent disease process. Examination of the tissues and content of the major portions of the gastro-intestinal tract and of the other organs by the mouse passage test led to only one isolation of the agent. This

infective material was found in the jejunum of one turkey sacrificed on the fifth day and was detected in the second mouse passage of the original material. Since gross evidence of disease was lacking in the first five days of the test, it was decided to follow the course of the infection in the rest of the birds by serologic tests and by attempts to isolate the disease agent from the blood stream. The gradual spread of ornithosis throughout the group of 14 test birds and 5 contact control birds offered an opportunity to study the epizootiology of the disease (table 2). Examination of suspect excreta indicated *when* the virus was present in the environment, and subinoculation of serially collected blood into mice indicated *when* each bird experienced viremia. Tests on sera collected at the same time indicated the degree of serologic response. It was seen that clinical disease did *not* follow oral feeding of the virus directly, as was the case with the airborne tests. Signs of disease and viremia appeared in most of the test birds and contact control turkeys 2 weeks after the virus was found in a single sample of excreta on the floor of the turkey runways and 4 weeks after the oral dose. Serologic response, as measured by the ICF test, was noted in each bird during or after viremia, never before.

From the virus isolation and serologic data, it was reasoned that a small nucleus of birds became infected by oral feeding of the virus and excreted it, but that rapid spread of virus to unaffected test birds and contact control turkeys occurred by aerogenic means.

Six turkeys in the test were sacrificed at various intervals to determine the status of the infection in terms of gross pathology and presence of virus in their tissues. These birds were chosen for sacrifice on the basis of the date of their last known blood stream infection. Gross lesions of infection were readily apparent in two birds, 14 and 41 days after blood stream invasion, yet the agent was not isolated from the pericardial sac, liver, spleen, kidney, gonads or cloacal contents. On the other hand, infective particles were isolated from the pericardial sac of two apparently healthy turkeys examined 35 and 54 days after blood infection. These

TABLE II

Results of NJI virus isolation from blood and ICF* tests on serum of turkeys exposed to ornithosis via the digestive tract

| Day after infection | 13 | | 23 | | 35 | | 45 | | 55 | | 97 | 153 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Turkey number | Viremia | ICF Titer | Viremia | ICF Titer | Viremia | ICF Titer | Viremia | ICF Titer | Viremia | ICF Titer | ICF Titer | ICF Titer |
| 119 | + | 16p | 0 | 16 | 0 | 32 | 0 | 32 | Sacrificed | | | |
| 132 | + | 4p | 0 | 4p | 0 | 0 | 0 | 8 | 0 | 8 | | |
| 135 | + | 0 | 0 | 4p | 0 | 0 | 0 | 8p | 0 | 8 | 2p | 4 |
| 120 | 0 | 0 | + | 0 | + | 8p | 0 | 16 | 0 | 32 | 64 | 2 |
| 127 | 0 | 0 | + | 2p | 0 | 64 | 0 | 128 | 0 | 128 | | |
| 134 | 0 | 0 | + | 0 | 0 | 128 | 0 | 16 | 0 | 8 | 32 | 16 |
| 128 | 0 | 0 | + | 0 | 0 | 128 | 0 | 128 | 0 | 64 | 64 | |
| 121 | 0 | 0 | + | 0 | 0 | 32 | 0 | 64 | 0 | 64 | 128 | 8 |
| 131 | 0 | 0 | + | 0 | 0 | 4p | 0 | 4 | 0 | 4 | 16 | 32 |
| 197 | 0 | 0 | 0 | 0 | + | 256p | 0 | 256 | Sacrificed | | | |
| 136 | 0 | 0 | 0 | 0 | + | 64 | 0 | 32 | 0 | 256 | 32 | 2 |
| 124 | 0 | 0 | 0 | 0 | + | 0 | 0 | 64 | 0 | 128p | 2p | 2 |
| 118 | 0 | 0 | 0 | 0 | + | 0 | 0 | 64p | 0 | 2p | 128 | 2p |
| 117 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Sacrificed | | | |
| Contact Control Turkeys (Uninoculated) | | | | | | | | | | | | |
| 199 | 0 | 0 | + | 0 | + | 64p | 0 | 2p | 0 | 8 | 4 | 2p |
| 198 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 128 | 8 |
| 175A | 0 | 0 | 0 | 0 | + | 32p | 0 | 8p | 0 | 2 | 4 | 2p |
| 137A | 0 | 0 | 0 | 0 | + | 0 | + | 256 | 0 | 128 | | |
| 200A | 0 | 0 | 0 | 0 | 0 | 0 | + | 256 | 0 | 128 | 32 | 16 |

* All indirect complement fixation tests were performed by Dr. B. Eddie, George Williams Hooper Foundation, University of California Medical Center, San Francisco. The ICF test results are expressed as the reciprocal of the highest dilution of serum exhibiting a complete reaction. Where no complete reaction was achieved on a serum, the reciprocal of the highest dilution exhibiting a *partial* reaction was recorded, e.g., 16p.

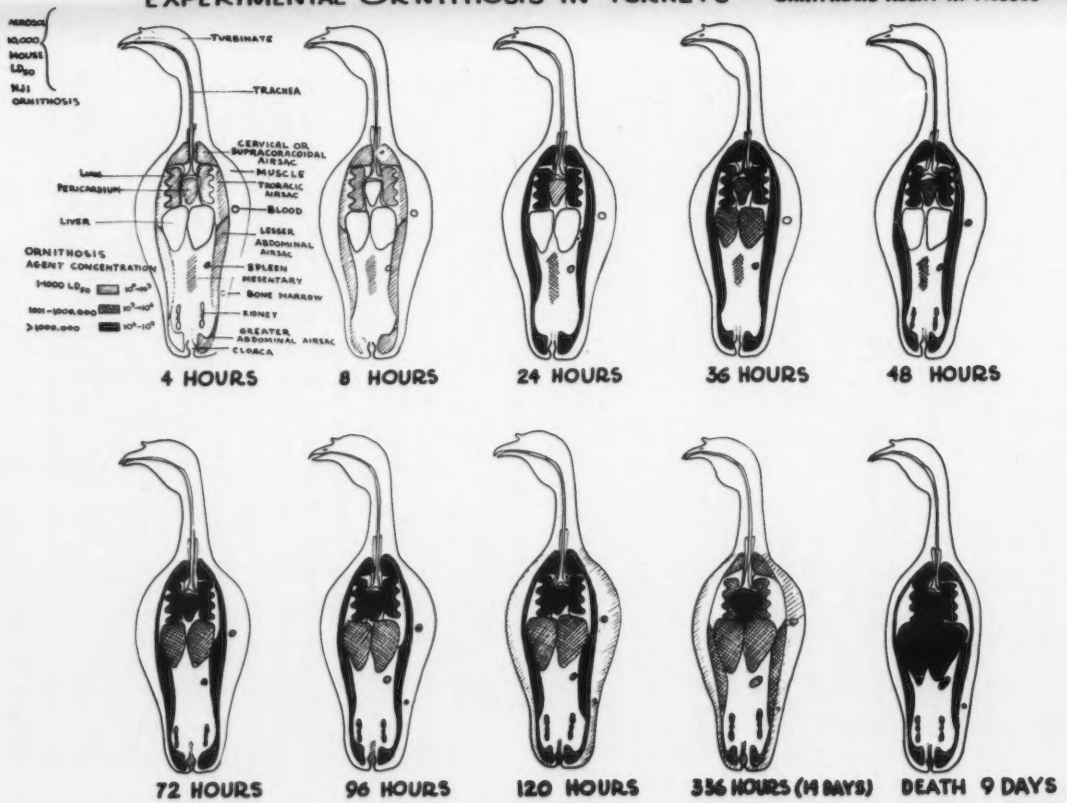


*Fig. 5.* Schematic representation of quantitative aerosol experiment. Each turkey diagram represents results on two turkeys.

unexplained isolations probably represent very rare occurrences in natural ornithosis.

In an effort to determine if physiological stress could cause a potential latent infection to become clinical disease, three turkeys infected with NJ1 virus two months previously were subjected to daily increasing doses* of cortisone acetate for eight days.

Samples of blood were collected on the 7th, 8th, 10th and 11th days after treatment. This blood was injected directly into mice for isolation of ornithosis agents. Two weeks after treatment, 2 of the three birds were sacrificed and their tissues examined for ornithosis infective particles by the mouse passage test. The third bird was sacrificed and examined 70 days after the last day of treatment. The mouse passage tests failed to indicate the presence of an ornithosis agent in the turkeys. ICF-detectable antibody titers remained relatively unchanged during and after the treatment. On the fourth and fifth day after the last injection, the sera of the turkeys contained an excessive amount of lipids.

## DISCUSSION

The studies reported herein suggest that acute, rapidly fatal ornithosis caused by the New Jersey strain results primarily from air-borne infection. That turkeys became diseased by the NJ1 isolate introduced by the oral route could not be conclusively shown. Two attempts to infect a total of 28 turkeys orally with doses which produced 100 percent clinical disease and 15 percent mortality by the air-borne route indicated that only a mild subclinical infection followed careful oral instillation of the agent. It is difficult to estimate what amount of infective material a turkey might ingest during an epizootic in a large group of birds so that no comparison between the amount given these experimental birds (40,000 to 340,000 mouse $LD_{50}$) and that ingested by an individual bird in a natural flock infection can be made. Droppings collected during several experiments and titrated in mice indicated the positive samples contained from 10 to 105,000 mouse $LD_{50}$ per gram excreta.

The findings in the quantitative aerosol experiment were interpreted in the following way: from the lung, where intensive multiplication of the agent took place, the organisms were picked up by the blood and filtered out in the liver, spleen and kidney. From the air sacs the organism was introduced into the pericardial membrane and epicardial surface and to the peritoneal serosa where the agent initiated an immediate inflammatory response. The continued multiplication of the organism at those sites further intensified the inflammatory reaction resulting in the congregation of large numbers of leucocytes and monocytes and heavy deposit of fibrinous exudate on the serous membranes and on the heart.

Throughout all the experiments conducted with the NJ1 ornithosis virus, turkeys surviving infection were examined for the presence of virus in their tissues. This work indicated that after the acute stage of the disease, virus could be recovered in the seventh week from the pericardial sac, but no isolation was made beyond the 54th day after experimental infection or viremia. It appeared that surviving birds recovered completely and eliminated the ornithosis organisms from their tissues. Autosterilization may play a significant role in the recovery of turkeys from ornithosis, as well as in the life history of a highly virulent, toxigenic disease agent.

These studies suggest the manner in which acute ornithosis may spread rapidly in a group of turkeys, but they shed no light on how the disease is initiated in a flock. Whether the turkey is an insidious carrier of ornithosis or only an incidental host to PLV agents excreted by other birds remains the subject of further experimentation.

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* from 12.5 mg/bird to 300 mg/bird intramuscularly

SUMMARY

The path of toxigenic ornithosis virus (NJ1 isolate) through turkeys exposed by the airborne and oral routes was traced by quantitative examination of tissues of affected birds.

Turkeys exposed to infective aerosols became acutely diseased and approximately 15 percent died. Titration in mice of tissue homogenates from infected birds killed at various stages of disease indicated that the virus multiplied primarily in the lungs, air sac system, and pericardium. High concentrations of the disease agent were found in the lung and air sac within 24 hours and were maintained until death. Blood stream infection occurred within 48 hours, whereupon the virus was distributed throughout the tissues. Concentration of virus in the pericardium increased gradually.

Birds surviving infection apparently sterilized their tissues over a period of 2 months. Qualitative examination by mouse passage tests of tissues of turkeys surviving infection indicated that up to 54 days after acute disease, the virus could still be isolated from the pericardium, but no isolations were made beyond the 8th week from a number of infected birds.

When the virus was fed orally, most birds showed no clinical effects nor significant serological response but excreted the agent into the environment and other birds became infected. It was reasoned that ornithosis infection may be initiated in a few birds by ingestion of infective material of extrinsic origin but the disease would spread rapidly among flock members if aerosols containing desiccated infected excreta were created.

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# THERMAL INACTIVATION STUDIES ON A TURKEY ORNITHOSIS VIRUS*

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IT has been known for a decade that ornithosis in turkeys and other meat birds, caused by viruses of the psittacosis-lymphogranuloma venereum (PLV) group, is a hazard to persons connected with the handling or processing of poultry. Since there is a potential risk of psittacosis to the consumer from infected processed birds, it is important to determine what tissues or organs of the processed infected fowl contain the virus. Quantitative studies on thermal death times or low temperature inactivation rates of ornithosis viruses were also desirable since this information could be used in the handling of infectious materials and in determining how the freezing process itself affects the viability of the viruses. A number of reports have appeared in the literature on the survival of viruses of the PLV group at various conditions of low temperature storage but none cite inactivation rates over an extended period.[1,6,9,10] Limited studies on the resistance of psittacosis organisms to heat have been reported, but these investigations did not offer quantitative data on the rate of destruction of the viruses by heat.[2,12,14]

## MATERIALS AND METHODS

For the studies on the effect of the commercial freez-

* This investigation was carried out under the sponsership of the Commission on Environmental Hygiene of the Armed Forces Epidemiological Board, and was supported (in part) by the office of the Surgeon General, Department of the Army.

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ing process on the viability of the ornithosis virus, seven turkeys which had been observed in a New Jersey processing plant to have lesions of ornithosis were sent via air express under dry ice refrigeration for examination. These birds were stored in a commercial deep freeze at temperatures ranging from –20C to –25C.

Individual birds were defrosted and examined for the presence of ornithosis viruses over a period of 372 days. The virus used in the thermal inactivation studies was isolated from the muscle of one of the New Jersey turkeys and was labeled the NJ1 isolate. Most isolations were made in 16 to 20 gram albino Swiss mice; however the tissue homogenates of three turkeys were injected into the allantoic fluid or yolk sac of embryonating chicken eggs as well as mice. The mouse proved to be the more reliable and sensitive isolation medium and was subsequently the animal of choice for isolation studies. The standard procedure for isolation of the turkey virus was to grind several grams of the tissue in question in a mortar with sand and beef heart broth (with antibiotics*, if contaminated by bacteria) to a final 20 percent suspension. The mixture was allowed to stand overnight or longer at 4C. and the supernatant fluid was inoculated in 0.5 ml amounts intraperitoneally into each of four mice. Mice dying or showing signs of illness within 15 days were examined for gross lesions of ornithosis and any suspicious exudate was stained by Macchiavello's method and examined microscopically for the presence of intracellular elementary bodies. Three blind passages were completed before any tissue sample was considered negative for ornithosis infectious particles.

The low temperature storage experiments were performed on samples of experimentally infected guinea pig tissues ground to a 20 percent suspension in broth and hermetically sealed in glass vials. The vials were stored either at –20C in a commercial deep freeze or in a dry ice chest

* A solution consisting of tyrothricin, 20 micrograms/ml; streptomycin H Cl, 250 micrograms/ml; and sodium sulfadiazine, 500 micrograms/ml; in beef heart broth.

where the temperature ranged from –38C to –65C. At approximately three-month intervals, a vial was removed from each storage place, defrosted at 5C and the contents assayed by the following technique. The suspension was shaken and decimal dilutions in beef heart broth were prepared. Each dilution and the initial suspension was injected in 0.5 ml amounts intraperitoneally into each of five mice. Death of the mouse within 21 days was chosen as the endpoint with the assumption that one infective particle of the highly toxic New Jersey isolate would multiply and ultimately kill the mouse. The number of infectious units present in any suspension was expressed in terms of mouse $LD_{50}$.

The freezing and thawing experiments were performed on a 10 percent mouse spleen and liver suspension of the NJI isolate which had been distributed into 9 × 125 mm Pyrex glass tubes. The tubes were hermetically sealed, cooled, and immersed in an isopropanol-dry ice bath at –65C. Thirty minutes later all the tubes were removed from the low temperature bath and placed in an ice bath at 1C and thawed for 10 minutes. Then one tube was removed for titration of its contents in mice. The remaining tubes were again frozen at –65C for 20 minutes and thawed in ice water for 10 minutes, and a tube removed for assay. The process was repeated three times. Similar Pyrex tubes were used for the thermal death time studies. Samples of a 20 percent mouse tissue suspension of the NJ1 isolate were distributed into the tubes which were then hermetically sealed and placed in an ice bath. At appropriate intervals, the tubes were removed from the ice bath and were placed in a circulating water bath at 56C ± 0.5C. An interval of 50 seconds was allowed for temperature equilibration, and then the tubes were removed at timed intervals, promptly cooled in the ice bath, and the contents titrated in mice. In the thermal death time determinations at tempertaures below 56C., equilibration time was not taken into account, nor was an ice bath used before or after the test, since the incubation period ranged from 1 to 12 days and the 50 second temperature equilibration time was insignificant. In the 37C determination, the tubes containing the

virus were stored in a 37C air incubator for the duration of the test. In the 20C test, the tubes were stored on the laboratory desk where the temperature was 20C. ± 2C.

## RESULTS

The seven processed turkeys stored at –20C. were examined over a period of 372 days, and 20 ornithosis virus isolations were made from tissues of these birds (Table 1).

TABLE I

Isolation of ornithosis agents from New Jersey turkeys stored at –20C

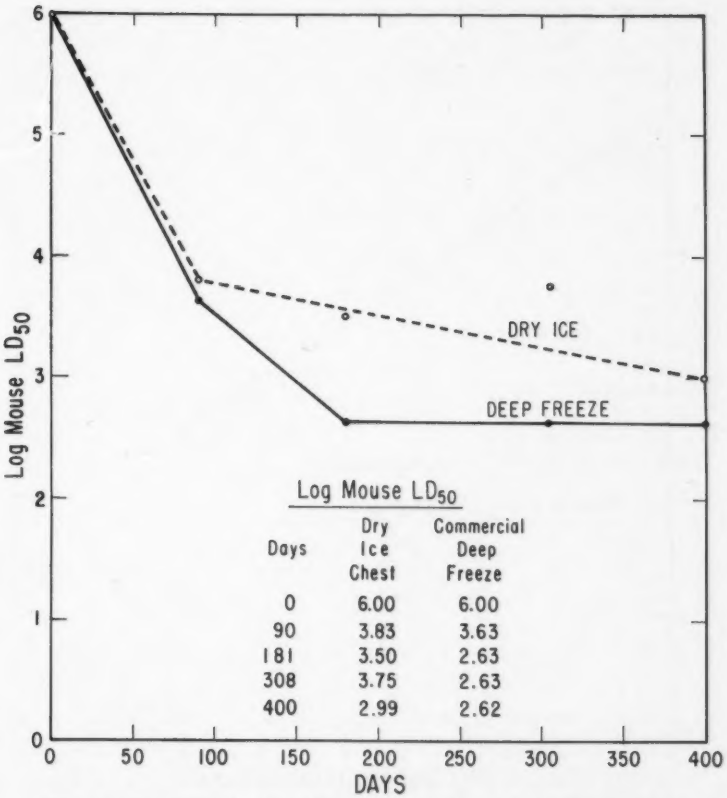
| Tissue examined | Results of isolation tests | | | | | | |
|---|---|---|---|---|---|---|---|
| | Turkey number* | | | | | | |
| | NJ1 | NJ2 | NJ3 | NJ4 | NJ5 | NJ6 | NJ7 |
| | Days of storage at —20C | | | | | | |
| | 18 | 50 | 78 | 100 | 200 | 310 | 372 |
| Spleen | + | + | 0 | | 0 | 0 | 0 |
| Liver | + | + | 0 | + | 0 | 0 | 0 |
| Kidney | + | + | 0 | + | 0 | 0 | + |
| Bone marrow | 0 | + | 0 | 0 | 0 | 0 | 0 |
| Pectoral muscle | + | 0 | 0 | 0 | | | |
| Thigh muscle | + | + | 0 | + | 0 | 0 | 0 |
| Pericardial exudate | + | + | | | | 0 | 0 |
| Peritoneal exudate | + | | | | | | |
| Air sac | | | 0 | + | 0 | 0 | 0 |
| Cloacal contents | | + | 0 | 0 | 0 | 0 | 0 |
| Heart | | | 0 | + | 0 | 0 | 0 |

* All turkeys had gross lesions of ornithosis in varying degree

The results summarized in figure 1 confirm many other isolated observations that the ornithosis viruses will survive prolonged storage in a sealed vial at temperatures of –20C or lower. In one instance, a psittacosis virus in 20C percent yolk sac suspension stored in a dry ice chest since 1948 still contained infective particles in 1955.[3]

Within 6 months after initial freezing and storage at –20C, a plateau in the rate of destruction of the virus was reached. At dry ice chest temperatures, the decline in numbers of infective particles continued to the last day of examination but always remained at higher levels than at –20C.

Alternate freezing and thawing over short periods of time had an obvious deleterious effect on the viability of this isolate as indicated in figure 2. Three freezing and thaw-




Log Mouse $LD_{50}$

| Days | Dry Ice Chest | Commercial Deep Freeze |
|---|---|---|
| 0 | 6.00 | 6.00 |
| 90 | 3.83 | 3.63 |
| 181 | 3.50 | 2.63 |
| 308 | 3.75 | 2.63 |
| 400 | 2.99 | 2.62 |

*Fig. 1.* Survival of the New Jersey turkey ornithosis isolate in 20 percent mammalian tissue suspension at temperatures below —20c.

ing cycles reduced the viable virus content 99.9 per cent. Extrapolation of the curve in figure 2 indicates that six or more cycles would destroy the infectivity of the suspension. These data also indicate that 21.5 percent of the lethal 50 percent units are destroyed in the initial freezing-thawing process. Applying this percentage to figure 1, the $LD_{50}$ would be reduced from 1 million ($1\times10^6$) to 785,000 ($1\times10^{5.89}$) from the effects of the initial freezing. This does not appreciably affect the general aspect of the curve in figure 1 between

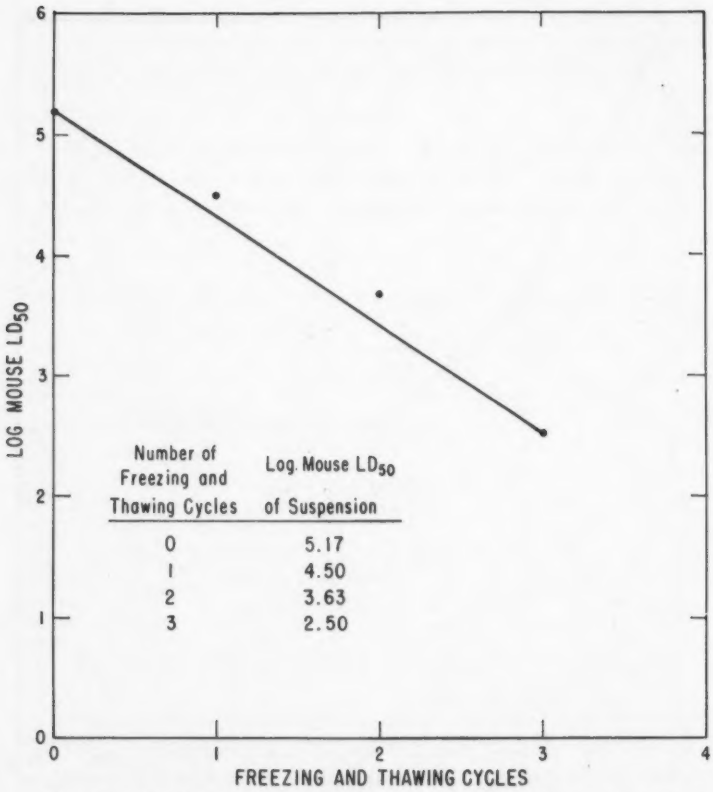



*Fig. 2.* Effect of rapid freezing and thawing on the survival of the New Jersey turkey ornithosis isolate in a 10 percent mammalian tissue suspension.

zero and 90 days. Thus at temperatures below –20C, inactivation of this ornithosis isolate is gradual rather than abrupt, and the rate does not drop sharply due to the first freezing.

From figures 3, 4 and 5, it is seen that the turkey ornithosis virus in 20 percent tissue suspension is relatively thermolabile. *Samonella typhosa,* for example, is killed after 23.8 minutes at 55C.[13] Extrapolation of the thermal destruction curves in figures 3, 4, and 5 indicate that at 56C, the virus is destroyed in 3.4 minutes; at 37C, approximately 27 hours; at

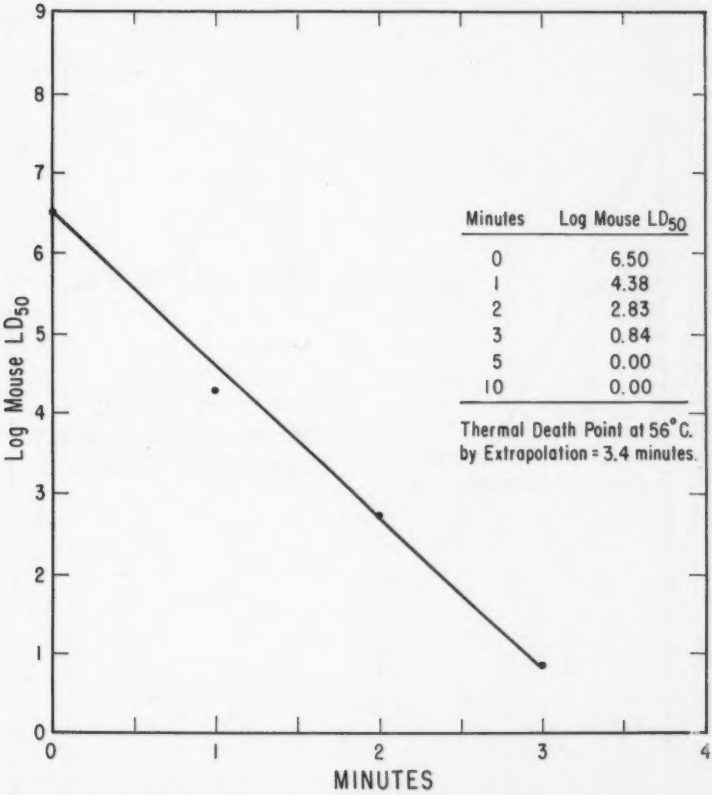



*Fig. 3.* Survival of the New Jersey turkey ornithosis isolate in 20 percent mammalian tissue suspension at 56C.

20C, approximately 10.4 days or 250 hours.

To ascertain that temperatures achieved inside the muscle and stuffing of a turkey during ordinary roasting were sufficiently high to inactivate ornithosis virus, a heat penetration experiment was conducted. Two turkeys of different weights were killed, dressed, stuffed, and roasted in the laboratory. Wire thermocouples, connected to a potentiometer outside of the oven, were inserted 2 to 3 inches

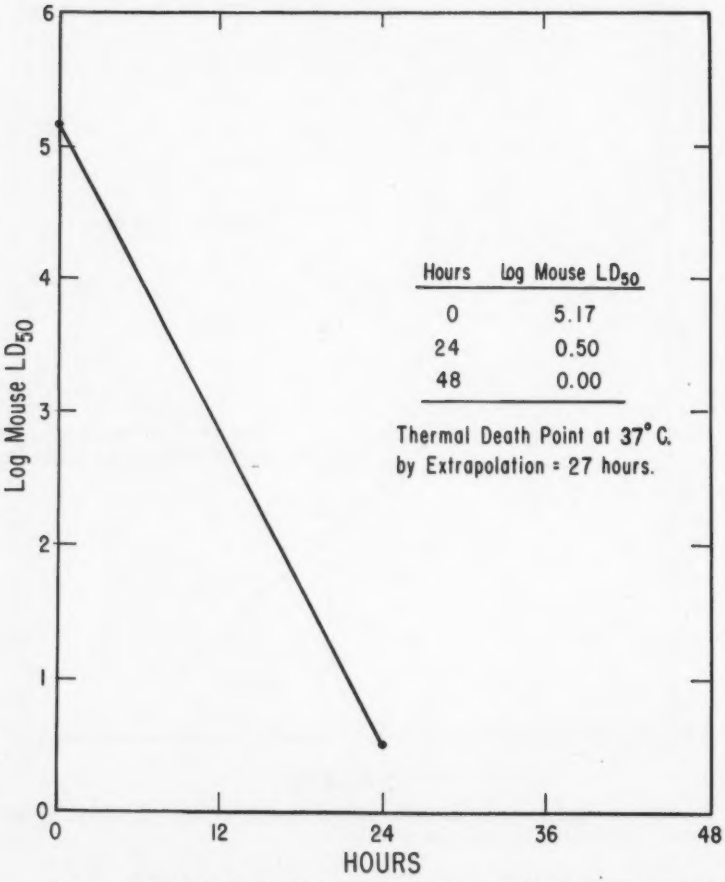



*Fig. 4.* Survival of the New Jersey turkey ornithosis isolate in 10 percent mammalian tissue suspension at 37C.

into the breast and thigh muscles and into the stuffing. The turkeys were roasted for the time recommended in a cookbook[5] according to the weight of the bird. Half-hourly potientiometer readings corrected to temperature Fahrenheit were made during the roasting period. Oven temperature

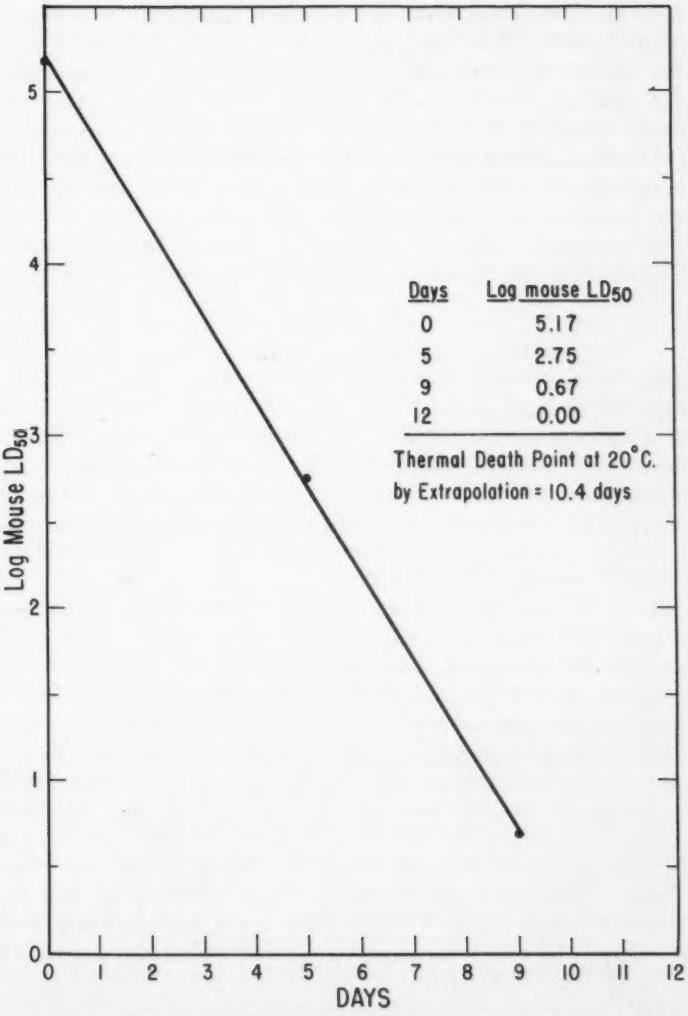



*Fig. 5.* Survival of the New Jersey turkey ornithosis isolate in 10 percent mammalian tissue suspension at 20C.

was also recorded from a mercury thermometer inserted into the oven. The data, presented in table 2, show that if the usually recommended roasting times and temperatures are followed, temperatures attained in the muscle meat are high enough to destroy all ornithosis and bacterial agents. In the most insulated portion of the roasted turkey, the stuffing, temperatures adequate to destroy ornithosis viruses rapidly are reached only after 2 hours. High temperatures for inactivation of ornithosis viruses are reached in the muscles 30 to 60 minutes sooner, depending on the weight of the bird.

## DISCUSSION

The isolation studies summarized in table 1 indicated that the ornithosis virus survived in frozen processed turkeys for as long as 372 days at –20C; thus present poultry processing procedures may not prevent the virus in infected fowl from reaching the consumer. Since the handling of infected meat cannot be considered to be without the risk of contracting psittacosis, adequate inspection of poultry during or before processing to eliminate suspect birds from consumer channels should be instituted.

As the storage time at –20C increased, fewer virus isolations were made. Gradual inactivation of viable virus could account for some loss, but other factors also influenced the isolation rate. Since the birds were naturally infected, the stage of the disease was not known. Autosterilization in some of the birds may have already begun. It was later shown that in ornithosis, the infective agent was widely disseminated in the turkey during the acute stage of the disease, but that in surviving birds the infection gradually receded and for a limited time the agent was found only in the kidney and pericardial membrane. After two months, virus could not be detected in any tissue.[11] The seventh New Jersey turkey examined at 372 days after processing and cold storage may have had an infection which at the time of killing had been localized in the kidney where there were sufficient numbers of infective particles for a few to survive the effects of low temperature storage.

TABLE II

Penetration of heat into muscles of turkey during oven roasting

| | Time, minutes | Oven temperature | Right breast | Left breast | Right thigh | Left thigh | Stuffing |
|---|---|---|---|---|---|---|---|
| Hen, 6.5 pounds, "ready to cook" weight | 0 | 140F | 87F | | 91F | 85F | 79F |
| | 30 | 270 | 139 | | 144 | 127 | 84 |
| Recommended* roasting time and temperature: 2.5-3 hours, 325F | 60 | 281 | 162 | | 168 | 160 | 98 |
| | 90 | 289 | 176 | | 180 | 175 | 122 |
| | 120 | 297 | 188 | | 196 | 182 | 150 |
| | 150 | 336 | 202 | | 209 | 197 | 172 |
| | 180 | 340 | 213 | | 217 | 207 | 189 |
| Tom, 11.5 pounds, "ready to cook" weight | 0 | 90F | 85F | 88F | 85F | | 75F |
| | 30 | 252 | 98 | 99 | 125 | | 80 |
| | 60 | 275 | 120 | 104 | 160 | | 94 |
| Recommended* roasting time and temperature: 4-5 hours, 275F | 90 | 286 | 139 | 139 | 185 | | 110 |
| | 120 | 279 | 155 | 152 | 198 | | 132 |
| | 150 | 280 | 167 | 163 | 206 | | 147 |
| | 180 | 284 | 175 | 174 | 209 | | 162 |
| | 210 | 284 | 183 | 183 | 212 | | 173 |
| | 240 | 284 | 192 | 192 | 213 | | 184 |
| | 270 | 284 | 196 | 196 | 214 | | 191 |

* "When You Roast Poultry" by Hilda Faust and Wilson E. Newlon; University of California, College of Agriculture, Agricultural Extension Service.

In the tests on 20 percent tissue suspensions of the ornithosis virus, the mouse lethal 50 percent unit titer dropped 99.95 percent after storage at –20C for six months but remained constant for at least another seven months. It appeared that the remaining organisms could be preserved indefinitely. A greater percentage of organisms survived when the infected material was stored under dry ice refrigeration. The mechanism for this is unknown but has been suggested to be due to a process called vitrification.[8]

While the thermal death time study was performed on a 20 percent mammalian tissue suspension of the ornithosis agent rather than on whole tissue, the data in this section indicated that definite, relatively rapid destruction of the agent in 10 to 20 percent tissue suspension takes place at 56C. Recent themal resistance studies by Enright *et al.*[4] indicated that the rate of destruction of *Coxiella burneti* suspended in whole raw milk at 143F (61.5C) was of a low order and that all the viable rickettsiae were not eliminated after 30 minutes at this temperature. Exposing the organisms to a temperature of 161F (71.5C) for 15 seconds, however, was adequate to eliminate viable *C. burneti.* The greater resistance to heat of *C. burneti* over the NJ1 ornithosis virus may be partially accounted for on the basis that the rickettsiae were suspended in whole raw milk rather than in broth-mascerated tissue suspensions. The high fat and protein content of milk proably exerts heat protective effects on viable microorganisms. Other factors, such as the pH or degree of dispersion of the infective particle, may seriously affect the resistance of microorganisms to heat.

The results obtained in the study on heat penetration in roasted turkeys compared favorably with those of Iacono *et al.*[7] indicating that temperatures sufficient to kill ornithosis organisms were achieved in the muscle of the roasted birds providing that ordinary cookbook recommended roasting time and temperature relationships were followed.

## SUMMARY

1. Turkeys naturally infected wih ornithosis and processed in a poultry processing plant and frozen, still contained viable ornithosis virus after 372 days of storage at –20C or below.

2. The titer of a 20 percent mammalian tissue suspension of turkey ornithosis virus stored at –20C or at dry ice chest temperatures for 6 months gradually dropped until 99.95 percent of the virus was nonviable, but the titer remained at a constant minimal level until the test was terminated 7 months later. At dry ice chest temperatures, the titer dropped less rapidly but the number of mouse lethal 50 percent units

was only slightly above that in the material stored at –20C at the end of the test.

3. At 56C the virus in 20 percent mammalian tissue suspension was destroyed in less than 5 minutes; at 37C, less than 48 hours; at 20C, less than 12 days.

4. Turkey roasting tests indicated that temperatures sufficient to kill ornithosis organisms were achieved in muscles of roasted birds if normal cookbook-recommended times and temperatures were followed.

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# A SLIDE SMEAR TECHNIQUE FOR THE DIAGNOSIS OF LARYNGOTRACHEITIS

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Received 14 April 1958

OSKAR Seifried[2] in 1931 was the first to describe, in detail, the histopathology of laryngotracheitis (LT) in chickens. In his excellent study, he stated that "characteristic intranuclear inclusions in the epithelial cells of the trachea are present in many cases," and that "in natural and experimental cases with involvement of the eyes, the eyelids show the same inflammatory process as the trachea." In such cases the writer has found the characteristic, acidophilic, intranuclear inclusions in the conjunctival epithelium. These inclusions were regularly found by Seifried in the earlier stages of the disease (as early as twelve hours post-inoculation) before advanced epithelial necrosis and massive exudation had occurred.

Twenty-five years passed before Siefried's findings in LT were used as a diagnostic tool. Recently Pirozok, Helmboldt and Jungherr[1] devised a "rapid diagnostic technique for laryngotracheitis" by means of examination of microscopic sections prepared by embedding tissues in "carbowax" a solid, polyethylene glycol. This method has the advantage of obviating the use of test birds, thus saving several days wait for results. Herein is described a simple, rapid, micro-slide technique for examination of appropriate tissues for the characteristic intranuclear inclusion bodies. Such inclusion bodies are seldom found in *all* affected birds *at the time of autopsy.*

However, our experience indicates that when a satisfactory flock sample, including several, live, sick and freshly dead birds, is submitted for examination, one or more specimens will have inclusions if laryngotracheitis is present. Many diagnosticians, not having the equipment or skilled assistance required for the preparation of histologic sections, could employ the slide smear technique with little addition to their present facilites.

## METHODS

*Slide-smear technique:* Selection of specimens and site for making the scraping is very important. Live birds showing early symptoms are best. These should be sacrificed in a manner to avoid introducing fresh blood over the tissues to be examined. Tissues from decomposing carcasses are unsuitable for microscopic examination. The mucosa of the larynx is often a favorable site. With a round blade scalpel, scrape off an intact sheet of epithelium including as little blood and exudate as possible. Deposit the scraping near the mid-point of a scrupulously cleaned, oil-free slide. With a second clean slide, press the tissue upon the first slide with a gentle, rotary pressure until it is flattened out in a thin layer. Draw the two slides apart parallel to one another. The object is to obtain smears containing numerous islands of intact epithelial cells toward the middle of the slide. In cases with conjunctivitis, smears are also made from one or more conjunctiva. One should make smears from at least three suitable birds.

Fix three to five minutes in absolute methyl alcohol.

Stain in Geimsa solution (one drop of stock per ml of distilled water) in Coplin jar 2 hours at 37 C, or overnight at room temperature.

Wash in tap water.

Differentiate in absolute methyl alcohol (three to five quick dipping motions) until the smear just retains a magenta cast. Wash quickly in tap water, air-dry and examine. If the stain is too light or too dark, the de-staining of the second slide can be adjusted.

In smears from LT affected birds the cytoplasm of virus-

infected epithelial cells stains lighter than normal—a pale blue with the cell walls often indistinguishable. Nuclear walls of such cells are thickened and stain a dark purple, usually with very little chromatin material in their interiors. The inclusion bodies themselves are purple with a pinkish cast, the bodies varying in shape from spherical to sausage shape or even triangular or bilobate. They often take up one-fourth to one-third of the nucleus. They are distinguished from the dark purple nucleoli by their larger size and pinkish cast. Another

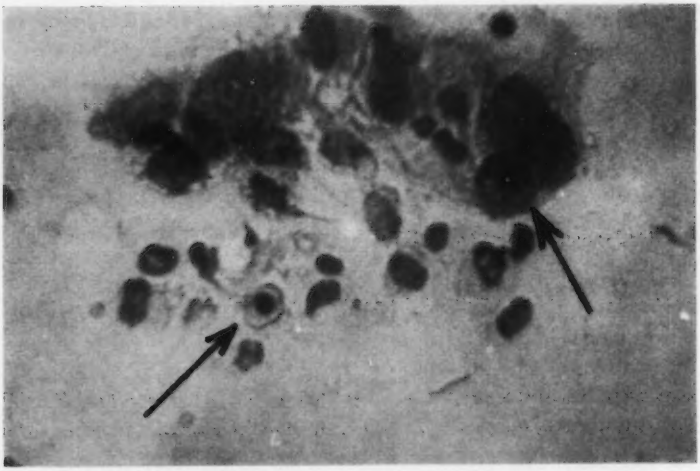

*Fig. 1.* Smear of conjunctival epithelial cells with intranuclear inclusions. Note halo around dark inclusion bodies. Giemsa stain, 900X.

distinguishing feature of the viral inclusions is that they are usually "framed" in a more or less well defined halo. Experience with the staining technique, with the appearance of the inclusions and persevering examination will enable one to distinguish these often when poor clearing has left a slight stain over-film or the smear is otherwise difficult to read.

Erythrocytes and leucocytes can usually be readily distinguished from the epithelial cells in smears as the latter usually can be found in contiguous groups and also by their contrasting staining and morphology.

In order to become familiar with the histopathology of the disease, we inoculated two groups of six birds each with .05 ml each of a commercial vaccine suspension, instilled intraocularly and intratracheally respectively. At intervals of 2, 4, and 6 days post-inoculation, two birds from each group with the most pronounced symptoms were sacrificed and examined. Tissue sections and smears of tracheal and conjunctival epithelium from these birds and normal controls were studied.

## RESULTS

The first symptoms to appear about forty-two hours post-inoculation were mild conjunctivitis (ocular group) and gasping (tracheal group). Symptoms throughout were relatively mild with no mortality. Post-mortem lesions were confined to a conjunctivitis and a mild, muco-catarrhal inflammation of the larynx and the adjoining one to two inches of the trachea.

Intranuclear inclusions were seen in tissues of birds from both the ocular and tracheal groups sacrificed 48 hours post-inoculation. At this stage, although the symptoms could only be described as respiratory in nature but not characteristic of any disease, the finding of inclusions made possible a specific diagnosis. Up to four days post-inoculation, inclusions in sacrificed birds were numerous in both conjunctival and tracheal epithelium and were easily recognized. These were still found on the sixth day in tracheas but were fewer and harder to distinguish in the birds sacrificed at that time.

Following this preliminary study, we began to submit all routine field cases of suspected laryngotracheitis to both slide-smear examination and to vent inoculation of susceptible birds with suspect exudate. Observations were made on the 3rd, 4th, and 5th days for vent reactions.

Of 52 field cases subjected to parallel tests, to date, 49 gave identical results with both tests. In one of the remaining three cases, inoculation results proved to be correct and the smear test in error when further check with virus challenge was done. We were unable to check the other two cases by virus challenge.

In field outbreaks, the writer has often seen well marked LT inclusions in smears taken from birds in the early clinical

stages when only non-specific conjunctivitis or a slight gasping with mild, catarrhal inflammation of the larynx were present.

## DISCUSSION

This technique requires no special equipment other than a microscope. It can be completed in as little as two hours, or at most, overnight, and no test birds are required. Even after gaining considerable experience in the smear technique, the writer has occasionally encountered a case of clinically "atypical" LT in which he felt it wise to check smear findings by other methods. Thus far, the smear technique has proven consistently reliable.

With few exceptions, qualitative laboratory techniques in disease diagnosis are best considered and used as aids, rather than complete answers. Their effectiveness depends on the experience and judgment of the user. The writer feels that the slide smear technique can be developed and used effectively as a rapid, routine diagnostic test for laryngotracheitis, in all cases where satisfactory autopsy specimens are available. Furthermore, a specific diagnosis can be made in the very early clinical stages of the disease.

Almost certainly the staining technique can be improved, especially the clearing step and possibly fixation of the smear. Other stains may prove more suitable than the Geimsa.

## SUMMARY

A slide smear technique is described for the diagnosis of laryngotracheitis wherein tracheal and conjunctival epithelial cells are smeared on a slide and stained with Giemsa stain. When suitable specimens are examined, intranuclear inclusion bodies characteristic of laryngotracheitis are readily detected. The accuracy of this method compared very favorably with that of the slower technique of vent inoculation of live birds with suspected material.

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# SALMONELLA IN ANIMALS: A REPORT FOR 1957

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Received 16 Aug 1958

IN 1957, 850 Salmonella cultures which were isolated in the United States from animal sources were typed serologically at the Communicable Disease Center, Chamblee, Georgia. There were 155 additional cultures belonging to the Arizona group, making a total of 1005 cultures in these related groups of animal pathogens.

By far the largest number of Salmonella cultures (622) came from poultry. Of these, 326 were from turkeys, 193 from chickens, and 8 from geese; in the case of the remaining 95 cultures, the senders failed to state the species from which the cultures were isolated. For the past twenty years it has been recognized that, taking Salmonella of animal origin as a whole, a disproportionately high number come from poultry. In 1939, Edwards[1] regarded fowls as the greatest reservoir of Salmonella infection in the United States. In a 15 year study of the occurrence and distribution of Salmonella types, Edwards, *et al.*[2,3] found that approximately half the cultures studied were isolated from fowls. Furthermore, the majority of the cultures were obtained from acute, fatal infections of

* Acknowledgement is made to Dr. P. R. Edwards in whose laboratory, at the U.S. Public Health Service, Communicable Disease Center, Chamblee, Georgia, these cultures were typed, and to Mrs. Carolyn Ramsey and Miss Mary Alyce Fife who shared in the work of serotyping the cultures. The author is with the Laboratory Services of the Animal Disease Eradication Division, ARS, USDA, and has been assigned to the CDC laboratory since April 15, 1957.

young birds. If the situation has changed at all since then, it is only in the increasing attention being given to carrier birds, particularly to culturing the intestines and internal organs of reactors to tests with *S. pullorum* and *S. typhimurium* antigens.

Table 1 summarizes the Salmonella types isolated from animals or food products of animal origin in 1957, together with the sources of the cultures. Since no comprehensive report on Salmonella types isolated from animals in the United States has been published since the 1948 report of Edwards *et al.*[2], comparisons with it are inevitable. However, the incidence of the various types fluctuates from year to year, a fact which does not show up in a summary of fifteen years' work. Accordingly, care must be taken in comparing the incidence of any one type in the two reports. Even after these allowances have been made, some points of interest in this regard remain:

1. *S. typhi-murium* remains the most common Salmonella type occurring in turkeys, and remains nearly four times as common in turkeys as in chickens.

2. *S. heidelberg*, of which there was not one culture listed in the 1948 report, comprised 8 percent of the total chicken cultures in 1957, 4 percent of the total turkey cultures, and 8.4 percent of the combined totals of chicken, turkey and the unspecified poultry cultures.

3. *S. infantis*, which had never been found in fowls in 1948, is likewise ascending in incidence and now comprises 6.7 percent of the cultures from chickens.

4. Although *S. pullorum* comprised only 3.9 percent of the total cultures from chickens, no conclusions should be drawn from these figures on the incidence of *S. pullorum* in fowls in the United States. Probably relatively few cultures of this organism, which is readily identified in the laboratories where it is isolated, are forwarded to a typing center.

5. *S. cholerae-suis* variety *kunzendorf* remains the most common Salmonella organism occurring in swine and, among animals, is largely confined to this species.

| Salmonella | Turkeys | | Chickens | | "Poultry" See text | | Geese |
|---|---|---|---|---|---|---|---|
| | O | C | O | C | O | C | C |
| 1. S. alachua | 1 | 2 | | | | | |
| 2. S. albany | 1 | 2 | | | | | |
| 3. S. anatum | 15 | 19 | 4 | 11 | 1 | 2 | |
| 4. S. bareilly | 2 | 2 | 4 | 6 | | | |
| 5. S. berta | | | | | | | |
| 6. S. binza | 3 | 3 | | | | | |
| 7. S. blockley | 1 | 1 | 1 | 1 | | | |
| 8. S. blukwa | | | | | | | |
| 9. S. bovis-morbificans | | | | | | | |
| 10. S. braenderup | 3 | 4 | | | 1 | 1 | |
| 11. S. bredeney | 5 | 11 | 1 | 2 | | | |
| 12. S. budapest | 1 | 1 | | | | | |
| 13. S. california | 1 | 1 | | | | | |
| 14. S. cambridge | 2 | 5 | | | | | |
| 15. S. chester | 3 | 3 | | | 2 | 3 | |
| 16. S. cholerae-suis var. kunzendorf | | | | | | | |
| 17. S. cubana | 2 | 2 | | | 1 | 1 | |
| 18. S. denver | | | | | | | |
| 19. S. derby | 3 | 4 | 2 | 3 | | | |
| 20. S. detroit | | | | | | | |
| 21. S. enteritidis | 2 | 12 | 3 | 3 | 1 | 3 | |
| 22. S. florida | | | | | | | |
| 23. S. gallinarum | 4 | 13 | 11 | 16 | 3 | 3 | |
| 24. S. georgia | | | | | | | |
| 25. S. give | 5 | 6 | 1 | 1 | | | |
| 26. S. hamilton | 1 | 1 | | | | | |
| 27. S. heidelberg | 9 | 14 | 13 | 16 | 11 | 22 | |
| 28. S. illinois | | | | | | | |
| 29. S. indiana | | | | | 1 | 1 | |
| 30. S. infantis | 7 | 9 | 13 | 13 | | | |
| 31. S. java | 1 | 4 | | | 1 | 4 | |
| 32. S. javiana | 4 | 4 | | | | | |

TABLE I Salmonella Cultures from Animal Sources: 1957 (In the colu

| Geese | | Pigeons | | Quail | | Other Birds | | Sheep | | Horses | | Cows | | Hogs | | Dogs | | Cats | | Skunks | | Seals | | Mink | | Nutria | | Rats | | Mice | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C | | C | | C | | C | | C | | C | | C | | O | C | C | | C | | C | | C | | C | | C | | C | | C | |
| | | | | | | | | | | | | | | 1 | 1 | | | | | | | | | | | | | | | | |
| | | | | | | | | 1 | | | | | | | | 1 | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | 1 | 4 | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | 49 | 72 | | | | | 1 | | | | | | | | | | | |
| | | | | 4 | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | 4 | |
| | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | 7 | | | | | | | | | | | | | | | |

TABLE I Salmonella Cultures from Animal Sources: 1957 (In the column H

| Pigeons | Quail | Other Birds | Sheep | Horses | Cows | Hogs | | Dogs | Cats | Skunks | Seals | Mink | Nutria | Rats | Mice | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C | C | C | C | C | C | O | C | C | C | C | C | C | C | C | C | |
| | | | | | | 1 | 1 | | | | | | | | | |
| | | | | | | | | 1 | | | | | | | | |
| | | | 1 | | | | | | | | | | | | | |
| | | | | | | 1 | 4 | | | | | | | | | |
| | | | | | | 49 | 72 | | | 1 | | | | | | |
| | 4 | | | | | | | | | | | 1 | | | | |
| | | | | | | | | | | | 1 | | | | 4 | |
| | | | | | | | | 1 | | | | | | | | |
| | | | | | | | | 1 | | | | | | | | |
| | | | | | | | | 2 | | | | | | | | |
| | | | | | | | | 7 | | | | | | | | |

lumn headings "O" = outbreaks, and "C" = cultures)

| Mice | Guinea Pigs | Monkeys | Gorillas | Reptiles | Sloth | Horse Meat | Dried or Frozen Eggs | Fish Meal | Liver Powder NF | "Processed Poultry By Products" | Total Cultures | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C | C | C | C | C | C | C | C | C | C | C | | |
| | | | | | | | | | | | 2 | 1. |
| | | | | | | | | | | | 2 | 2. |
| | | | | | | 2 | | | 1 | | 35 | 3. |
| | | 1 | | | | | | | | | 9 | 4. |
| | | | | | | | | 1 | | | 1 | 5. |
| | | | | 1 | | | | | | | 5 | 6. |
| | | | | | | | | 2 | | | 2 | 7. |
| | | | | 1 | | | | | | | 1 | 8. |
| | | | | | | | | | | | 1 | 9. |
| | | | | | | | | | | | 5 | 10. |
| | | | | | | | | 1 | | 1 | 5 | 17. |
| | | | | | | | 1 | | | | 1 | 18. |
| | | | | | | 5 | | | 1 | | 18 | 19. |
| | | | | 1 | | | | | | | 1 | 20. |
| 4 | 1 | | | | | | | | | | 24 | 21. |
| | 3 | | | | | | | | | | 3 | 22. |
| | | | | | | | | | | | 32 | 23. |
| | | | | | | 2 | | | | | 2 | 24. |
| | | | | | | | 1 | | | | 9 | 25. |
| | | | | | | | | | | | 1 | 26. |
| | | | | | | | | 1 | | | 53 | 27. |
| | | | | | | | | | | | 1 | 28. |
| | | | | | | | | | | | 3 | 29. |
| | | | | | | | | | | | 22 | 30. |
| | | | | | | | | | | | 15 | 31. |
| | | | | | | | | | | | 4 | 32. |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 14. | S. cambridge | 2 | 5 | | | | | | | |
| 15. | S. chester | 3 | 3 | | | 2 | 3 | | | |
| 16. | S. cholerae-suis var. kunzendorf | | | | | | | | | |
| 17. | S. cubana | 2 | 2 | | | 1 | 1 | | | |
| 18. | S. denver | | | | | | | | | |
| 19. | S. derby | 3 | 4 | 2 | 3 | | | | | |
| 20. | S. detroit | | | | | | | | | |
| 21. | S. enteritidis | 2 | 12 | 3 | 3 | 1 | 3 | | | |
| 22. | S. florida | | | | | | | | | |
| 23. | S. gallinarum | 4 | 13 | 11 | 16 | 3 | 3 | | | |
| 24. | S. georgia | | | | | | | | | |
| 25. | S. give | 5 | 6 | 1 | 1 | | | | | |
| 26. | S. hamilton | 1 | 1 | | | | | | | |
| 27. | S. heidelberg | 9 | 14 | 13 | 16 | 11 | 22 | | | |
| 28. | S. illinois | | | | | | | | | |
| 29. | S. indiana | | | | | 1 | 1 | | | |
| 30. | S. infantis | 7 | 9 | 13 | 13 | | | | | |
| 31. | S. java | 1 | 4 | | | 1 | 4 | | | |
| 32. | S. javiana | 4 | 4 | | | | | | | |
| 33. | S. kentucky | 3 | 6 | 1 | 1 | | | | | |
| 34. | S. litchfield | 1 | 2 | | | | | | | |
| 35. | S. manhattan | 4 | 4 | 1 | 1 | | | | | |
| 36. | S. meleagridis | 13 | 36 | | | | | | | |
| 37. | S. montevideo | 1 | 2 | 3 | 3 | 1 | 1 | | | |
| 38. | S. muenchen | 2 | 5 | 1 | 9 | 2 | 2 | | | |
| 39. | S. new-brunswick | | | | | | | | | |
| 40. | S. newington | 2 | 2 | 1 | 5 | | | | | |
| 41. | S. newport | 13 | 21 | 2 | 2 | 3 | 4 | | | |
| 42. | S. norwich | | | | | | | | | |
| 43. | S. oranienburg | | | 3 | 4 | | | | | |
| 44. | S. orion | 1 | 1 | 1 | 1 | | | | | |
| 45. | S. panama | 3 | 3 | | | | | | | |
| 46. | S. pullorum | 4 | 7 | 53 | 77 | 9 | 12 | | | |
| 47. | S. reading | 1 | 2 | | | | | | | |
| 48. | S. saint-paul | | | 1 | 1 | 7 | 12 | | | |
| 49. | S. san-diego | 10 | 19 | 1 | 1 | | | | | |
| 50. | S. schwarzengrund | 5 | 6 | 1 | 1 | 2 | 2 | | | |
| 51. | S. senftenberg | 1 | 1 | | | | | | | |
| 52. | S. siegburg | | | | | | | | | |
| 53. | S. stanley | 1 | 1 | | | | | | | |
| 54. | S. tennessee | 1 | 5 | 1 | 1 | 1 | 1 | | | |
| 55. | S. thompson | 7 | 7 | 1 | 1 | | | | | |
| 56. | S. typhi-murium | 43 | 73 | 8 | 13 | 18 | 19 | 8 | 7 | |
| 57. | S. typhi-suis | | | | | | | | | |
| 58. | S. urbana | | | | | | | | | |
| 59. | S. virginia | | | | | 1 | 1 | | | |
| 60. | S. worthington | | | | | | | | | |
| 61. | 6,8:d | | | | | 1 | 1 | | | |
| 62. | 3,10:1,6 | | | | | | | | | |
| 63. | 28:y | | | | | | | | | |
| | | 192 | 326 | 132 | 193 | 67 | 95 | 8 | 7 | |

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| | | | | | | 1 | 4 | | | | | | | | |
| | | | | | | 49 | 72 | | | 1 | | | | | |
| | 4 | | | | | | | | | | | 1 | | | |
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| | | | | | | 2 | 2 | | | | | | | | |
| | | | | | 2 | | | | | | | 3 | | | |
| 7 | | 3 | 6 | 2 | | 4<br>1 | 4<br>1 | | 1 | | | 3 | 1 | 1 | |
| | | | | | | | | 5 | | | | | | | |
| 7 | 5 | 3 | 7 | 2 | 2 | 60 | 86 | 18 | 1 | 1 | 2 | 9 | 1 | 1 | |

| | | | | | | | | | | | | | | | |
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| | 4 | | | | | | | | | | | 1 | | | |
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| | | | | | | 2 | 2 | | | | | 1 | | | |
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| | 1 | | | | | | | | | | | 1 | | | |
| | | | | | | | | 1 | | | | | | | |
| | | | | | | 2 | 2 | | | | | 3 | | | |
| 7 | | 3 | 6 | 2 | | 4<br>1 | 4<br>1 | | 1 | | | 3 | 1 | 1 | 1 |
| | | | | | | | | 5 | | | | | | | |
| 7 | 5 | 3 | 7 | 2 | 2 | 60 | 86 | 18 | 1 | 1 | 2 | 9 | 1 | 1 | 5 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 1 | | 1 | 5 | 17. |
| | | | | | | | 1 | | | | 1 | 18. |
| | | | | | | 5 | | | 1 | | 18 | 19. |
| | | | | 1 | | | | | | | 1 | 20. |
| 4 | 1 | | | | | | | | | | 24 | 21. |
| | 3 | | | | | | | | | | 3 | 22. |
| | | | | | | | | | | | 32 | 23. |
| | | | | | | 2 | | | | | 2 | 24. |
| | | | | | | | 1 | | | | 9 | 25. |
| | | | | | | | | | | | 1 | 26. |
| | | | | | | | | 1 | | | 53 | 27. |
| | | | | | | | | | | | 1 | 28. |
| | | | | | | | | | | | 3 | 29. |
| | | | | | | | | | | | 22 | 30. |
| | | | | | | | | | | | 15 | 31. |
| | | | | | | | | | | | 4 | 32. |
| | | | | | | | | 1 | | | 8 | 33. |
| | | | | | | | | | | | 2 | 34. |
| | | | | | | | | | | | 6 | 35. |
| | | | | | | | 1 | | | | 39 | 36. |
| | | | | | | | | | | | 6 | 37. |
| | | | | | | | 2 | 2 | | | 20 | 38. |
| | | | | | | | | | | | 1 | 39. |
| | | | | | | 3 | 3 | 2 | | | 15 | 40. |
| | | 1 | | | 1 | 1 | | | | | 32 | 41. |
| | | | | | | 6 | | | | | 6 | 42. |
| | | | | | | | | 4 | | 1 | 10 | 43. |
| | | | | | | | | | | | 2 | 44. |
| | | | | | | | | | | | 5 | 45. |
| | | | | | | | 1 | | | | 102 | 46. |
| | | | | | | | | | | | 2 | 47. |
| | | 2 | | | | | | | | | 15 | 48. |
| | | | | | | 1 | | | | | 21 | 49. |
| | | | | | | | | 4 | | | 13 | 50. |
| | | | | | | | | 6 | | | 7 | 51. |
| | | | | | | | 1 | | | | 1 | 52. |
| | | | | | | | | | | | 1 | 53. |
| | | | | | | | | 3 | | | 10 | 54. |
| | | | | | | | | | | | 8 | 55. |
| 1 | 1 | 2 | | | | | | | | | 144 | 56. |
| | | | | | | | | | | | 1 | 57 |
| | | | 1 | | | | | | | | 1 | 58. |
| | | | | | | | | | | | 1 | 59. |
| | | | | | | | | 1 | | | 1 | 60. |
| | | | | | | | | | | | 1 | 61. |
| | | | | | | | | | 1 | | 1 | 62. |
| | | | | | | | | | | | 5 | 63. |
| 5 | 5 | 6 | 1 | 2 | 1 | 20 | 10 | 28 | 4 | 2 | 850 | |

There were 64 cultures from food products of animal origin: 20 from horse meat, 10 from dried or frozen eggs, 28 from fish meal, 4 from N. F. liver powder and 2 from "processed poultry by-products" for use in animal feeds.

The presence of Salmonella in eggs is easily explained, even expected. They can be accounted for either through ovarian infection of the parent hen or contamination with intestinal material after the shell has been formed. References to the isolation of Salmonella from eggs and egg products abound in the literature and the variety of types is noteworthy. It is not known whether the comparatively small number of cultures received from this source in 1957 represents a slackening of interest in this already well-explored field. Once food material, contaminated from whatever source, has been through a processing plant, the plant equipment may be contaminated and remain so, contaminating subsequently processed materials for some time, unless steps are taken to correct the situation.

Salmonella organisms are not commonly found in horses. *S. abortus-equi* has all but disappeared in the United States, and there remain only occasional gastro-intestinal infections in foals, usually caused by *S. typhi-murium*. The presence of Salmonella organisms in commercial chopped horse meat almost certainly is due to plant contamination, the source of which could be humans, rodents, cats, or dogs. The same sources of contamination may account for the presence of Salmonella in fish meal, for the organisms are not found in the fish themselves. The presence of these pathogens in this material, as well as in the "processed poultry by-products," either of which may be used in the manufacture of poultry feed, suggests that there needs to be a definitive answer to the question of whether the further processing of the feed does in fact guarantee the absolute elimination of these organisms.

There is a growing tendency to cite poultry and poultry products as the source of Salmonella infections in humans. Undoubtedly many such infections do occur. However, addi-

tional consideration should be given to the prevention of human contamination of animal feeds. It would be highly desirable to set the same sanitary standards for the preparation of animal feeds which are already in effect for food intended for human consumption.

Table 2 summarizes the serological analysis of the 155 members of the Arizona group received from animal sources. A total of 138 cultures were isolated from turkeys and were confined to two types, namely 7:1, 7, 8 and 7:1, 2, 6. The number of each one of these is approximately equal to the number of *S. typhi-murium* cultures from turkeys, and combined is nearly twice as great. These organisms were not frequently submitted from chickens in 1957.

TABLE II

Arizona cultures from animal sources: 1957
(In the column headings "O" = outbreaks, and "C" = cultures)

| Arizona | Turkeys | | Chickens | | "Poultry" See text | | Other birds | | Mink | | Monkeys | | Sheep | | Reptiles | | Fish meal | | Total cultures |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | O | C | O | C | O | C | O | C | | C | | C | | C | | C | | C | |
| Arizona 5:1, 2, 5, 6 | | | | | | | | | | | | | | | | | | 1 | 1 |
| 5:13, 15 | | | | | | | 1 | | | | | | | | | | | | 1 |
| 7:1, 2, 6 | 45 | 70 | | | 1 | 3 | | | | | | | | | | | | | 73 |
| 7:1, 7, 8 | 33 | 68 | 1 | 1 | 1 | 1 | | | 1 | | | | | | | | | | 71 |
| 9a, b:17, 20 | | | 1 | 1 | | | | | | | | | | | | | | | 1 |
| 10:1, 2, 5 | | | 1 | 2 | | | | | | | | | | | | | | | 2 |
| 13:1, 2, 5 | | | | | | | | | | | | | | | 1 | | | | 1 |
| 18:13, 14 | | | 1 | 1 | | | | | | | | | | | | | | | 1 |
| 26:23-30 | | | | | | | | | | | 1 | | | | | | 1 | | 2 |
| 26:30 | | | | | | | | | | | | | 2 | | | | | | 2 |
| TOTAL | 78 | 138 | 4 | 5 | 2 | 4 | 1 | 1 | 1 | | 1 | | 2 | | 1 | | 2 | | 155 |

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# CASE REPORT—EQUINE ENCEPHALOMYELITIS IN PHEASANTS IN FLORIDA

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*Agricultural Experiment Station, Gainesville, Florida State Board of Health, Jacksonville and Florida Livestock Board, Dade City, Florida*

Received Aug 14 1958

THE first report of naturally occurring cases of equine encephalomyelitis in pheasants was published by Tyzzer, *et al.*[6] Subsequently, cases were reported from New Jersey,[1,2,3,9] Rhode Island,[7,8] and Pennsylvania.[4] This paper describes an outbreak of eastern equine encephalomyelitis in ring-necked pheasants in Florida. The diagnosis was made on the basis of clinical, pathological, and virological findings.

Seven, 14 week-old pheasants, five of which were living, were submitted to the laboratory in August, 1957. The owner reported that of a total of 140 pheasants confined to pens according to age groups, 18 to 20 had died and that deaths had been restricted to birds 14 weeks of age. Older pheasants were not affected. Clinically the birds were lethargic, droopy, ataxic, and paralyzed. For the most part, no gross lesions were noted at necropsy.

Brains from sick pheasants were fixed in 10 percent neutral formalin, sectioned at six microns, and stained with hematoxylin and eosin for histopathological study. Brains for viral studies were frozen immediately upon collection and stored until examined.

Histological examination of brains revealed a nonsuppurative encephalitis. There was substantial perivascular

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† Florida State Board of Health
* Florida Livestock Board
Florida Agricultural Experiment Station; Journal Series, No. 774.

cuffing with lymphocytes (fig. 1), thickening of the walls of blood vessels, leptomeningeal infiltration with lymphoctes, and neuronal degeneration.

Clinical observations and microscopic findings permitted a diagnosis of encephalitis of undetermined etiology. Viral isolation studies were initiated to determine the causative agent. Frozen brains were triturated in a Tenbroeck grinder

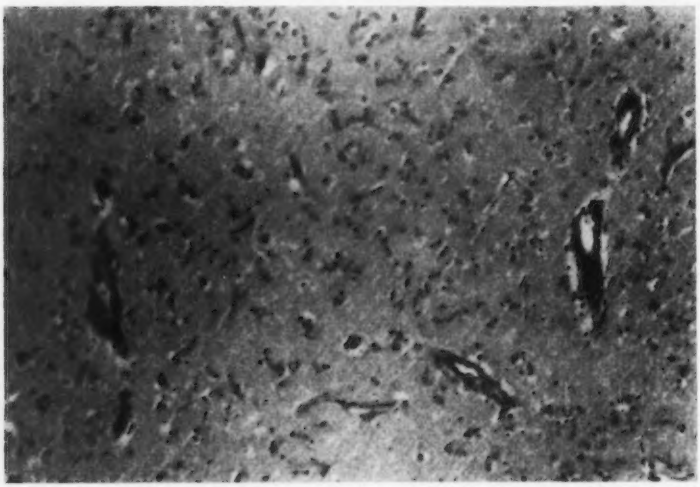

*Fig. 1.* Photomicrograph of pheasant brain showing perivascular cuffing with lymphocytes. Hematoxylin and eosin, 423X.

after sufficient buffered saline was added to make a 1:10 suspension by weight. The suspension was centrifuged at 2,000 rpm for 10 minutes and the supernatant used to inoculate embryonated chicken eggs after the addition of penicillin and streptomycin at the rate of 10,000 units and 50 mg. per ml., respectively. Ten, seven day-old embryonated eggs which were inoculated by the yolk sac route, using 0.1 ml of inoculum, died in 25 to 30 hours showing extensive hemorrhages. Seven passages were made by the same route and in all cases embryo mortality duplicated the initial inoculation results. Yolk material harvested from dead embryos was bacteriologically sterile. Young, white mice, inoculated intracerebrally with 0.03 ml of supernatant material from ground

pheasant brains, died about 96 hours post inoculation. Stupor and paralysis occurred in mice shorty before death.

The titer of the isolate contained in yolk sac material was determined. Embryonated eggs were inoculated by the yolk sac route with 0.1 ml of serial tenfold dilutions of yolk material. Five eggs were used per dilution. The chick embryo $LD_{50}$ of the agent, calculated according to the method of Reed and Muench,[5] was $10^{6.8}$ per 0.1 ml of yolk material.

Serum neutralization tests with eastern and western equine encephalomyelitis antisera in mice were conducted against the isolate. On the basis of the high neutralizing index (3200) with eastern encephalomyelitis antisera, the agent isolated from the pheasant brains was believed to be the virus of eastern equine encephalomyelitis.

## SUMMARY

1. An outbreak of eastern equine encephalomyelitis in pheasants is described.

2. Histopathological findings and clinical observations suggested a presumptive diagnosis of viral encephalitis.

3. Identification of the agent involved in this disease outbreak was made serologically.

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Dr. R. C. Klussendorf
Veterinary Medical Service
Commercial Solvents Corporation
Terre Haute, Ind.

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**1** UNIVERSITY OF GEORGIA FEEDING TRIAL

| Treatment (40 birds per treatment) | Antibiotic Per Ton of Feed (grams) | Av. Wt. 8 Weeks (lbs.) | Feed Conversion |
|---|---|---|---|
| BACIFERM (zinc bacitracin) | 4 | 2.61 | 2.13 |
| Penicillin | 4 | 2.58 | 2.12 |
| BACIFERM-PB* | 4 | 2.69 | 2.23 |

*contains zinc bacitracin plus penicillin (3:1 ratio)

**2** UNIVERSITY OF MASSACHUSETTS TEST

| Treatment (32 birds per treatment) | Antibiotic Per Ton of Feed (grams) | Average Male Wt. 8 Wks. (lbs.) | Lbs. Feed per Lb. Gain | Mortality (each treatment 32 birds started) |
|---|---|---|---|---|
| Control | 0 | 3.26 | 2.15 | 1 |
| BACIFERM (zinc bacitracin) | 4 | 3.34 | 2.16 | 0 |
| BACIFERM (zinc bacitracin) | 6 | 3.38 | 2.11 | 1 |
| Penicillin | 4 | 3.37 | 2.11 | 2 |

**3** ALABAMA POLYTECHNIC INSTITUTE TEST (No. 2)

| Treatment (40 birds per treatment) | Antibiotic Per Ton of Feed (grams) | Av. Wt. 7 Wks. (lbs.) | Lbs. Feed per Lb. Gain |
|---|---|---|---|
| BACIFERM (zinc bacitracin) | 4 | 2.60 | 2.00 |
| Penicillin | 4 | 2.53 | 2.04 |

**4** LEADING NORTHEASTERN UNIVERSITY

| Treatment (100 birds per treatment) | Antibiotic Per Ton of Feed (grams) | Av. Wt. 9 Weeks (lbs.) | Lbs. Feed per Lb. Broiler |
|---|---|---|---|
| Control | 0 | 3.43 | 2.44 |
| BACIFERM (zinc bacitracin) | 4 | 3.49 | 2.40 |
| Penicillin | 4 | 3.40 | 2.40 |

**5** FEED MANUFACTURER IN SOUTHEAST

| Treatment (200 birds per treatment) | Antibiotic Per Ton of Feed (grams) | Av. Wt. 66 Days (lbs.) | Lbs. Feed per Lb. Gain | Cost per Lb. Meal |
|---|---|---|---|---|
| Penicillin | 4 | 3.18 | 2.31 | 14.73¢ |
| BACIFERM (zinc bacitracin) | 4 | 3.19 | 2.30 | 14.66¢ |
| BACIFERM-PB* | 5 | 3.19 | 2.30 | 14.70¢ |

*contains zinc bacitracin plus penicillin (3:1 ratio)

**6** COMMERCIAL TEST FARM BROILER TRIAL

| Treatment (1000 birds per treatment) | Antibiotic Per Ton of Feed (grams) | Av. Wt. 9 Weeks (lbs.) | Lbs. Feed per Lb. Gain |
|---|---|---|---|
| BACIFERM (zinc bacitracin) | 4 | 3.2 | 2.48 |
| Penicillin | 4 | 3.2 | 2.48 |

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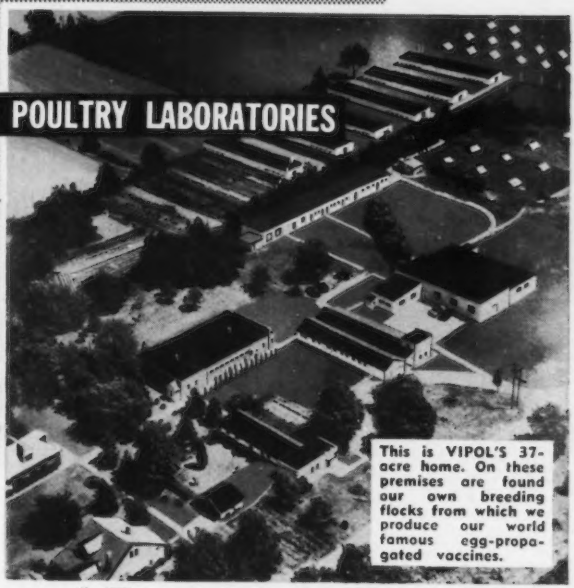

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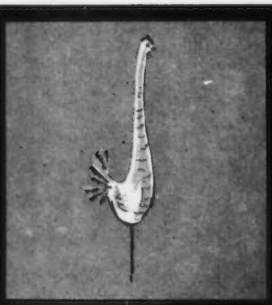

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During the rainy, humid weather of 1958, many flocks were hard hit by coccidiosis caused by *E. acervulina*. Meanwhile 100 million other chicks and growing birds suffered no serious loss. They were raised on Unistat-medicated feed.

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